Development of sensing systems for environmental monitoring

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ABSTRACT

This project focused on the investigation and the development of a chemical sensing system for the determination of chromium Cr^{6+} and a bio-reactor followed by electrochemical detection at a glassy carbon electrode, for the determination of organochlorine compounds.

The conjugation of Cr^{6+} with 1,5-diphenylcarbazide was studied at various types of electrodes such as glassy carbon, ultra-trace epoxy-graphite, chemically or un-modified carbon-paste and dropping-mercury. The cyclic voltammetric behaviour of the complex was also investigated.
In addition, the possibility of developing a chemical sensor, i.e. an electrochemical probe capable of sensing Cr6+ through its complexation with 1,5-diphenylacarbazide was studied.

The conjugations of 1-chloro-2,4-dinitrobenzene, 2,4-dichloronitrobenzene and ethacrynic, which are electrophilic organochlorine compounds, with reduced glutathione, were studied in order to test the bioreactor developed, based on the immobilisation of glutathione s-transferase. This was carried out at different types of electrodes such as glassy-carbon, gold, silver, platinum, epoxy-graphite, hanging-mercury, and ferrocene-modified rotating-disc electrodes.

TABLE OF CONTENTS

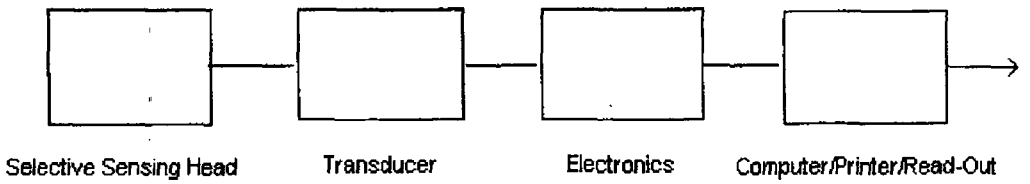
CHAPTER 1.0

SENSORS FOR HEAVY METALS MONITORING

1.1 What is a Sensor ? :

A sensor can be described in different ways, such as a '*device for sensing*', or a '*device using a sensing process such that information on material(s) tested can be obtained*' [1]. This is a device that converts a physical, chemical, or biological change usually into an electrical signal, which may be relayed to a computer, integrator or printer [2] (Figure 1.1).

Figure 1.1 Sensor Schematic



The different types of sensors can be classified as *physical*, *chemical* or *biological* (biosensors), depending on their characteristics :

i) Physical Sensors, which measure a physical change occurring (resulting from a chemical reaction) in the sample such as temperature, pressure, volume, displacement, density, or mass;

ii) Chemical Sensors, which measure a chemical change occurring in the sample such as pH, concentration, chemical composition; and

iii) Biological Sensors or *Biosensors*, which measure a chemical and/or biological change occurring in the sample such as pH, concentration, chemical composition. This is done by coupling the bio-component with either a chemical or physical transducer. The biologically-sensitive part of this device can either be one or a few enzymes, an organelle, a membrane component, a bacterial cell or a cell, an antibody or an antigen, or even plant or mammalian tissues.

Sensing processes are based on the exchange of energy, matter or energy and matter between the sample and the sensing device. The sensor response time is dependent on various factors, such as pressure and temperature, as well as by convection and diffusion.

The sensor response should ideally be limited to analyte concentration.

The main sensing processes used for the development of sensors are electrochemical, thermal, spectrophotochemical, mass spectrochemical or radiochemical transducers :

i) Electrochemical, where the electroactivity of the sample is monitored, yielding an information about the species present in the sample. This is obtained by measuring the change in potential of an electrochemical cell through specific interaction at an electrode/solution interface. The change in current can also be monitored, depending on the mass transport of species to and from an electrode surface;

ii) *Thermal*, based on the measure a temperature change; and

iii) *Spectroscopic*, based on the measure of absorbance, reflection, refraction, transmittance, or emission [3].

Sensors have been of interest for a wide range of applications (Table 1.1), from the glucose oxidase sensor designed for diabetics, to environmental, clinical, or even military purposes.

Table 1.1 Examples of Sensor Applications

Application Areas	**Examples**
Clinical and Medical	Glucose [4-8], Dopamine [9], Blood Banks, HIV, Hepatitis B, TBC [10].
Industrial	Process Monitoring [11, 12], Drug or Food Processing, Quality Control, Contaminants Monitoring.
Environmental	Water, Air and Soil Monitoring (pesticides, pH, nitrates, nitrites), BOD Testing.
Veterinary	Animal Diseases, Quality Control in meat.

1.2 Amperometric electrochemical detectors

Most amperometric electrochemical sensors use a three electrode system (reference, working and auxiliary) in which a fixed potential difference is applied between the working electrode and the reference electrode. This applied potential difference speeds up the sensing process by speeding up the redox reaction at the surface of the working electrode.

It is the current generated at the working electrode which is amplified and plotted as a function of time : any current generated by redox reaction is plotted and a peak is observed on the recording device. The potential applied at the working electrode is set against the reference electrode.

1.2.1 Reference electrodes

Different reference electrode have been developed and used with time, the most important being the standard hydrogen electrode, the calomel electrode and the silver/silver chloride. Other reference electrodes of lower importance have also been developed such as the mercury/mercurous or the hydrogen/palladium electrode. All electrode potentials are measured relative to the Standard Hydrogen Electrode, which has a potential of 0.00 V.

The *standard hydrogen electrode* was the first to be developed, and many standard half-reaction redox potentials of metals present in handbooks were obtained using this type of electrode. It is however very impractical and cumbersome, so that other types of electrodes have been developed since.

The *silver/silver chloride* electrode is a so-called 'wet' electrode of the silver metal/silver insoluble salt type. In a flow-through system, it is generally placed downstream from the working electrode, making electrical contact with the electrolyte via a micro-porous plug.

$$AgCl\ (s) + e^- \leftrightarrows Ag\ (s) + Cl^-$$

The main disadvantage of this type of reference electrode over the other types is that it requires to be placed far away from the working electrode, as excessive noise, instability and non-linear response would arise from multiple junction potentials and ohmic (iR) drops. Another drawback of this type of electrode as a reference comes from the charging currents produced at the solution/electrode surface when a potential is applied to the working electrode. Such currents decay exponentially, taking a long time before reaching zero, and involve a considerable analytical down-time if this detector is turned off. A solid silver wire coated with silver chloride has been recently studied as reference electrode, but revealed to be fairly unstable and increase the system noise as well as the corrosion of the system.

The *calomel electrode*, based on mercury/mercurous chloride, is similar to the Ag-AgCl, but suffers many of this latest drawbacks, as well as using highly toxic salts.

$$HgO\ (s) + H_2O + 2e^- \leftrightarrows Hg\ (liq) + 2OH^-$$

Another reference electrode, based on mercury/mercury sulphate is based on a similar reaction.

$$HgSO_4 + 2e^- \leftrightarrows Hg\ (liq) + SO_4^{2-}$$

A novel type of reference electrode, called *hydrogen/palladium reference electrode* was designed to overcome the drawbacks from the silver/silver chloride electrode. It is a very small electrode that can be placed close from the working electrode, is maintenance free, stable, and not easily poisoned.

1.2.2 Working electrodes

Working electrodes will only function properly within a specific window dictated by the material of the electrode (Table 1.2) as well as the pH of the electrolyte. For example, the electrolysis of many compounds on a glassy-carbon working electrode can be done up to approximately + 1.3 V vs. Ag-AgCl without experiencing difficulties.

However, potentials of + 5.0 V vs. Ag-AgCl or higher applied to this same glassy-carbon working electrode would alter its surface or even destroy the electrode. Another limitation to the maximum reduction potential at which each electrode can be used comes from the excessive current due to oxygen present in the buffer, which gets reduced to water. It is however possible to overcome by nitrogen bubbling. Therefore, strong reduction potential will only be used if the dissolved oxygen is first removed from the electrolytes by nitrogen bubbling.

Table 1.2 Potential window of mercury, platinum, graphite, glassy-carbon and gold Working Electrodes under basic, neutral and acidic conditions (vs. Ag-AgCl) [13] :

Condition	Platinum (V)	Mercury (V)	Glassy Carbon (V)	Carbon (V)	Gold (V)
Acidic	+ 0.4 to -1.2	+ 1.3 to - 0.4	+ 1.3 to - 0.8	+ 1.5 to - 0.2	+ 1.2 to - 0.5
Neutral	+ 0.2 to - 1.8	+ 0.8 to - 0.7	-	+ 1.0 to - 1.3	+ 0.8 to - 1.1
Basic	0 to - 2.0	+ 0.5 to - 1.0	+ 0.6 to - 1.5	+ 1.7 to - 0.8	+ 0.7 to - 1.3

There are two different type of processes occurring at the interface between the electrode and the solution, the *faradaic* and *non-faradaic processes*.
The processes in which reduction and oxidation occur follow Faraday's law and are commonly called *faradaic processes*. In such cases, the magnitude of the current will be determined by the extent of redox reaction occurring.
Processes not involving electrolysis, such as adsorption or desorption, can occur at a potential at which charge transfer reactions do not occur (either thermodynamically or kinetically unfavourable), are called *non-faradaic processes*

(as they do not involve any electron-transfer).

Assuming the total current flowing through the cell is called i_T, and the faradaic and non-faradaic current are called i_F and i_{NF},

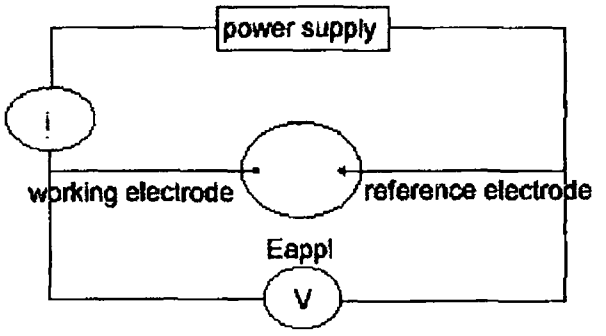
$$i_T = i_F + i_{NF}$$

1.2.3 Polarography - Definition and basics

Polarography is as an electrochemical technique invented in 1922 by Jaroslav Heyrovsky [14], based on the measurement of the current that flows in solution as a function of an applied voltage. The electric potential (commonly called voltage) is varied in a regular manner between two sets of electrodes (reference and working), while the current is monitored. This method is useful in detecting and determining substances simultaneously, and is applicable to relatively small concentrations (10^{-6} to 10^{-2} mol.l^{-1}). Polarography is a specific example of voltammetry.

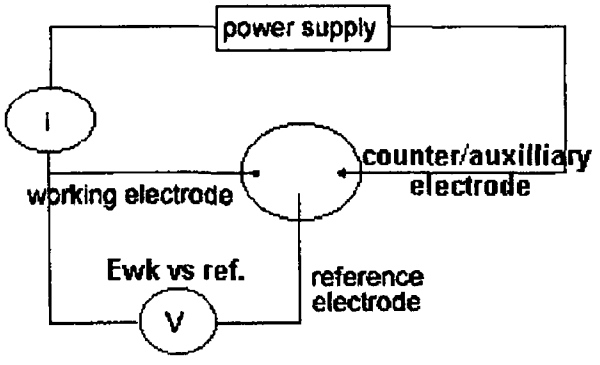
Voltammetry can be defined as *'an electrochemical technique in which a faradaic current passing through the electrolysis solution is measured while an appropriate potential is applied to the polarizable or indicator electrode'* [15].

Figure 1.2 Basic two-electrode voltammetric cell, appropriate for use in solutions of low resistance and micro-electrodes [16].



The voltammetric cells can either be two or three-electrode based (Figure 1.2 and Figure 1.3).

Figure 1.3 Three-electrode voltammetric cell [16]



In a polarographic or voltammetric cell, the decrease of the reactant concentration at the electrode surface during electrolysis is dependant on *diffusion*, *convection* and *migration* currents. During electrolysis, cations move towards the cathode and anions towards the anode as well as any species being oxidised or reduced if charged via convection or diffusion. Depending on the charge of the species, the mass transfer process of the electroactive species will be modified via migration, which current can be negative, zero or positive.

1.3 Stripping voltammetry for heavy metals monitoring

1.3.1 Introduction

Since the early twentieth century and its industrial revolution, human activities have modified natural cycles, causing regional and global redistribution of more or less hazardous elements in aquatic and terrestrial systems, up to critical concentrations in various cases. Several hundred thousand tons of heavy metals are dumped every year in aquatic systems [17], most of them non-biodegradable.
As a result, increasing environmental research developed detailed studies on the fate and variety of those elements, new technologies being introduced and optimised to monitor their concentration, especially for environmental and health purposes [18].
A quantity of metalloids and selected metals are most frequently analysed in both environmental and biological materials, as presently considered environmentally and toxicologically significant. They are Arsenic (As), Cadmium (Cd), Chromium (Cr), Iron (Fe), Manganese (Mn), Nickel (Ni), Lead (Pb), Antimony (Sb), Tin (Sn), Thallium (Tl), Uranium (U), Mercury (Hg) and Vanadium (V) [19].
These elements appear to be essential to many organisms in small doses whereas high doses adversely affect both human health and ecosystem.
They also appear in man, in food and in the environment in a wide concentration range from ultra-trace levels ($\mu g.l^{-1}$ to $ng.l^{-1}$), up to higher levels ($mg.kg^{-1}$ or $mg.l^{-1}$) in a few cases of anthropogenic pollution [19].

Up to now, these elements were mainly analysed by graphite furnace atomic absorption spectrophotometry (GF-AAS), flame atomic absorption (AA), inductively coupled plasma atomic emission spectrophotometry (ICP-AES), or mass spectrometry (ICP-MS). However, electroanalytical techniques have recently gained much interest due to their capability for miniaturisation, allowing on-site monitoring, lower costs, very low detection limits, multi-elemental determination and their capability for metal speciation [20].

For many of those elements, the redox speciation is a factor of discrimination between their labile or inert characteristics. It is also necessary for biological (bio-accumulation, bio-concentration, bio-availability, toxicity) or geochemical (transport, adsorption, precipitation) interpretations [21]. Speciation is important as metals present in different ionic forms have different environmental and toxicological effects. One form of the metal may be harmful, toxic or damaging to the environment, fauna, flora or human beings, while the other form may be harmless.

One of the oxidation states may be electrochemically active while the other may not be within the potential of the electrode. Electrochemically inactive (or with low reactivity) heavy metals species include As^{5+}, Cr^{3+}, Mn^{+4}, Sb^{5+}, Sn^{4+}, Tl^{3+}. However, these particular oxidation states can be, for certain metals, determined indirectly by polarography and adsorptive stripping voltammetry (AdSV), allowing for the discrimination of electroactive and electro-inactive species.

Another way of carrying out speciation studies is by using ligands which form selective and stable complexes with one oxidation states of the metals of interest, enabling the determination of whole species at this oxidation state.

The following species have been successfully discriminated [22] : Fe^{2+} & Fe^{3+}, Cr^{3+} & Cr^{6+}, Tl^{+} & Tl^{3+}, Sn^{2+} & Sn^{4+}, Mn^{2+} & Mn^{4+}, Sb^{3+} & Sb^{5+}, As^{3+} & As^{5+}, Se^{4+} & Se^{6+}, V^{4+} & V^{5+}, U^{4+} & U^{6+}.

1.3.1.1 Stripping Voltammetry

Generally, direct polarographic determination cannot be carried out at the sensitivity required for trace metals in environmental and biological samples and a stripping step is required.

Commonly either square-wave voltammetry (SWV) or differential pulse polarography (DPP) are used in conjunction with adsorptive, anodic or cathodic stripping voltammetry (AdSV). Adsorptive anodic and cathodic stripping voltammetry give lower detection limits ($ng.l^{-1}$) than direct polarography due to the pre-concentration process carried prior to detection.

Adsorptive stripping voltammetry is a technique based on the formation of a complex between the metal ion of interest and an organic ligand, and its adsorption onto the electrode surface prior to its cathodic or anodic stripping. The organic ligand must have affinity for the electrode surface [23].

The current resulting from the stripping process is directly proportional to the concentration of the metal ion. This technique requires that the complex formed should be stable, electrochemically active, as well as possessing kinetics allowing quick adsorption on the electrode surface.

In stripping analyses, any oxidation state can be collected, and the material is collected as a mono-molecular layer which is directly accessible for reduction/oxidation [24].

However, AdSV shows limited applications in the case of inland waters since it requires an electrolyte of high ionic strength, unless a supporting electrolyte is added. Another way to overcome this problem is by using micro-electrodes [25] (diameter in the micrometer range).

Stripping voltammetry can be used on carbon-paste electrodes, screen-printed carbon electrodes, carbon-disc micro-electrodes, and on mercury-film electrodes; However, most electrochemical methods developed for the analysis of foodstuffs or environmental samples are based on the traditional hanging or static mercury

drop electrodes (HMDE and SMDE) [26-31].

Solid electrodes have been developed in the last two decades to overcome the relative unpopularity of stripping voltammetry, mainly caused by the use of mercury, its toxicity and inconvenience involved, and its limited range of potentials for anode reactions.

Different solid electrodes were therefore developed and studied in stripping voltammetry, and they can be schematised as following [32] (Figure 1.4).

Figure 1.4 List of solid electrodes used in voltammetry

Solid Electrodes	- Metallic	- Thin Film	- Disposable
		- Micro-electrodes	- Disposable
Carbon Electrodes	- Carbon Paste		
	- Glassy Carbon		
	- Bulk		
	- Screen-Printed	- Disposable	
		- Long-lived	

Electrodes can be modified by different procedures to enhance their sensitivity and versatility towards specific analytes. This modification process can be either carried out in-situ or prior to the analysis.

In-situ, the modification can be obtained using mercury, gold or organic compounds. Prior to the analysis, the modification is obtained by surface coating using an insoluble salt, an insoluble gold salt, a biomaterial or a polymer film. This can be also be done introducing a mercury compound, a mediator system, or a biomaterial into the bulk.

1.3.1.2 Carbon-paste electrodes

The first carbon-paste electrode was developed and reported in 1958 by Adams [33]. This type of electrode is based on a conducting (i.e. fine carbon powder mixed with a hydrophobic solvent) or non-conducting binder [34] (also called pasting liquid).

Common pasting liquids are paraffin oil, silicon oil, nujol, ceresin wax, bromoform, or bromonaphtalene, with optimum ratios binder/graphite being from 1.8 ml/5 g paraffin to 2 ml nujol/3 g graphite [35].

This type of electrode is widely used as working electrode due to its low cost, conductivity and ease of use, which represent perfect characteristics for disposable single-use devices in potentiometry or voltammetry [36, 37].

In addition, carbon-paste electrodes can be used between - 1.4 to + 1.3 V versus saturated calomel reference electrode (SCE). This is a much wider range than attained by mercury electrodes. Unmodified carbon-paste electrode are also widely used for routine analysis, especially for stripping analysis of metals [38].

Most research on carbon-paste electrode has been applied on the development of chemically-modified carbon-paste electrodes. By judiciously modifying these carbon-paste electrodes, enhanced sensitivity and selectivity can obtained. The main reasons for chemical modification of a carbon-paste electrode are as that it may allow

1) The preferential pre-concentration of one components of the analyte over others,

2) The exploitation of catalytic electrochemical responses (using transition metals as modifiers),

3) The immobilisation of molecules involved in specific electrochemical reactions,

4) The alteration of the physical properties of the electrode surface.

For example, chemically-modified carbon-paste electrodes were developed following classical chemical reactions such as for the determination of Ni^{2+} [39, 40] or Cr^{6+} &Cr^{3+} [41, 42]. Two complexing agent, respectively dimethylglyoxime [43] and 1,5-diphenylcarbazide, were used for that purpose, thus forming two electrochemically active complexes that can be adsorbed onto the surface of the carbon paste prior to their stripping. A wide number of other examples on such electrodes are described in literature [41, 42, 44-49].

Modified carbon-wax composite electrodes have also been widely studied, based on the incorporation of quantities of modifiers into melted wax/graphite powders mixtures. The main advantages of this type of electrode offers over carbon-paste electrodes are the homogeneous dispersion of the modifier due to the melting and solidification processes, and their mechanically rigid surface [50].

In order to attain good performance, carbon-paste electrodes should possess the following features :

1) Electrochemically inertness over wide ranges of potentials,
2) Low background currents,
3) High oxygen and hydrogen evolution over-voltages,
4) High electrical conductivity and simple regeneration of the electrode surface [51].

The condition of the working electrode surface alters the current response in voltammetric measurements, so that specific preparation techniques are extremely important.

Surface regeneration of the electrodes is a detrimental parameter in the development of the electrode itself, and is usually complicated and irreproducible. Relative roughness appears to be an important parameter to achieve linear diffusion as a dominant mass transport by minimising the overlap of diffusion zones associated with the electroactive sites. Randomly spaced electroactive sites and non-electroactive particles define the model of roughness that is likely to inhibit such overlapping [52].

1.3.1.3 Glassy carbon electrodes

Electroanalytical methods employed on glassy-carbon electrodes use either electroplated thin mercury-films (MFEs) [21-23, 53-54], or chemically-modified electrode surfaces [55]. The use of plated thin mercury-film on the working electrodes enables the pre-concentration of the analytes in the mercury-film prior to their stripping so that extremely low detection limits down to ultra-trace ($\mu g.l^{-1}$ tong.l^{-1}) may be obtained [56].

However, the use of mercury is subject to electrode fouling, is expensive (an electrode used with a mercury-film can not be used for another task), requires careful handling, and is not environmentally friendly.

Alternatively, working electrodes can be chemically-modified with electron donating chelating compounds [57]. Ferrocene and its derivatives are commonly used as electrode modifying agents. The addition of such modifiers can also enhance or allow the species selection being achieved by simple chemical affinity.

1.3.1.4 Gold, micro-electrodes, over-oxidised polypyrrole film

Gold electrodes have also been widely studied in stripping voltammetry throughout the years, mainly due to the amalgamation characteristics that gold has with mercury [58]. However, it is not as cheap than carbon-paste for disposable sensor development, and the use of mercury is not environmentally friendly. It is possible to use this type of electrode in conjunction with a polymer film (e.g. polypyrrole [59]) by chemical modification.

Speciation of Cr^{6+} was obtained by Turyan and al.[60]) by assembling a monolayer of 4-(mercapto n-alkyl) pyridinium on gold electrodes. Disc gold electrodes and gold-film electrodes are now traditionally used for the determination of electropositive elements such as mercury, selenium, arsenic an copper.

The gold film can be occasionally deposited in-situ by electrolysis. The main drawback of this type of electrode is in the regeneration of their surface, as it was found that the reaction products can't be removed completely from the electrode during the electrode regeneration.

Applications of adsorptive stripping voltammetry were mainly focused on the study of saline samples since a high ionic strength electrolyte is required. Due to the need for in situ voltammetric probes for heavy metals, and especially in low ionic strength freshwater, Ir-based mercury-coated micro-electrodes were developed . The major advantage of such devices is in their size (μm range) which overcomes disadvantages linked to the ohmic drop in resistive solutions, and avoids the formation of inter-metallic species in mercury films.

The use of over-oxidised polypyrrole films have been studied for application in anodic stripping voltammetry [61], mainly due to their exhibition of cation - permselectivity. In that way, they prevent the matrix effects caused by organic/inorganic compounds based on a size-exclusion effect, letting the cation go through the membrane and concentrate on top of the electrode prior to their stripping. The main benefits of using such films for working electrodes are that they decrease electrode fouling and minimise sample pre-treatment.

1.3.1.5 Screen-printed electrodes

Recent years saw the growing needs for the development of decentralised analytical determination, especially for environmental and health purposes. The development of screen-printed electrodes (SPEs) offered the possibility to achieve such a task. So-called 'disposable' carbon electrodes are becoming more widely used year after year. They are manufactured at a very low-cost (30-40 pence per electrode) at large-scale, are easy to operate, are portable and reliable [62]. As carbon is inexpensive and conductive, such electrode substrate is ideal for an economic fabrication of disposable electrodes. Screen-printing is also an easy and simple process which gives reliable and reproducible surfaces.

Stripping-based tools or remote electrodes suitable for in-situ analysis offer the option for continuous environmental monitoring surveillance, as well as decentralised clinical and environmental testing since portable potentiostat were launched on the market in the last few years. Strips are usually based on planar carbon or gold working electrodes and a silver reference electrode, printed on an inexpensive plastic or ceramic support [63]. Sol-gel-derived gold electrodes have also been developed at a higher cost, obtaining characteristics such as favourable electron-transfer kinetics due to gold surfaces, as well as regeneration and bulk modification [64].

Noble metals or carbon based ink have been successfully studied and by incorporating electro-catalysts or chelating agents chemically-modified electrodes specific for particular analytes were successfully produced.
It is furthermore possible to purchase screen-printing equipment and inks, so that the preparation of SPEs is relatively simple, inexpensive, and highly versatile.
Screen-printed electrochemical sensors for decentralised testing of naturally occurring compounds, drugs or some substances of environmental and industrial importance have already been developed.

Wang J. and Tian B. describe a mercury-coated-screen-printed carbon electrode for the single-use decentralised testing of lead in either unstirred and non-dearated solutions (100 µl drops), obtaining a detection limit of 30 $ng.l^{-1}$ (ppt). Sensors for the amperometric determination of copper were also described.
Other publications described the development of methods using mercury micro-electrode where the mercury is deposited on glassy carbon, carbon-paste, Hg/Ir or Pt/Ir electrodes. Recent advances on the screen-printing market were discussed, as the use of metal-dispersed screen-printed carbon electrodes, as well as molecular imprinting.

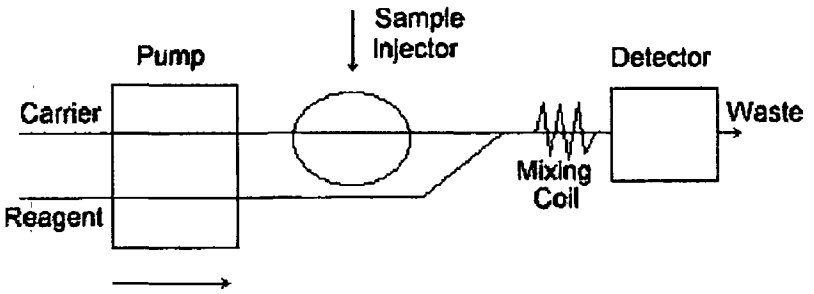
1.3.1.6 Flow-Injection analysis

The development of carbon-paste electrodes integrated to flow-injection analysis (FIA) systems has been the focus of a substantial amount of work, especially with the view of developing continuous *on-site* monitoring. Sample handling, storage and pre-treatment (if necessary) appear to be the main major sources of error in chemical analysis [65]. This is due to losses by adsorption or contamination, or speciation information that can be lost due to physical or chemical changes (temperature, pH, pCO_2, pH_2S, pO_2), occurring upon storage.

The first components of the first Flow Injection Analysis (FIA) were a peristaltic pump, an injector (disposable syringe with hypodermic needle), a flow-through 'air-gap' ion-selective electrode (ISE), a potentiometer, and a chart recorder (Figure 1.5).

FIA has rapidly developed with the proliferation of personal computers and the availability of automated, high-precision syringe pumps and valves. Although numerous papers and meetings have proved the versatility and advantages of Flow-Injection, and it is now widely used in research [67].

Figure 1.5 First type of FIA apparatus. This system was designed to monitor ammonium concentrations in effluents via its conversion to ammonia [66].



Future developments of Flow-Injection will probably concentrate on interfacing of FI with electro-thermal Atomic Absorption and Inductively Coupled Mass Spectrometer, combining Sequential Injection (SI) with Capillary Electrophoresis, and using beads as reagent carriers.

1.4 Study of the electrochemical reaction between 1,5-diphenylcarbazide and Cr^{6+} at a carbon-paste electrode

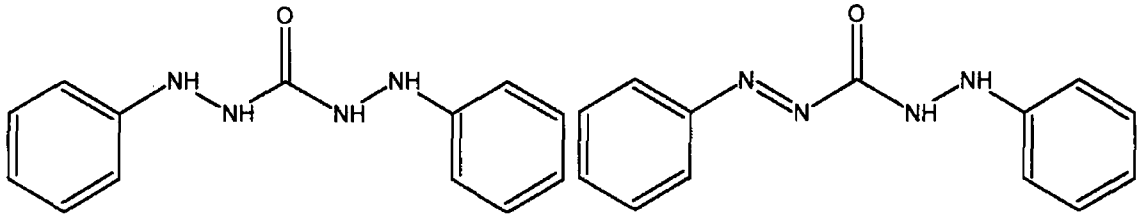
1.4.1 Conjugation reaction between hexavalent chromium and 1,5-diphenlycarbazide

1,5-Diphenylcarbazide (DPCI, Figure 1.6) is an organic compound commonly used in the determination of hexavalent chromium by ultra-violet spectrometry. A number of articles have been published on the study of the reaction between 1,5-diphenylcarbazide and Cr^{6+}, described as follows [68, 69]:

$$2Cr^{6+} + 3DPCI \quad <=====> \quad 2Cr^{3+} + 3\ DPCO + 6H^{+} \qquad (a)$$

$$Cr^{3+} + DPCO \quad <=====> \quad [CrDPCO] + 2H^{+} \qquad (b)$$

Figure 1.6 1,5-diphenylcarbazide and 1,5-diphenylcarbazone

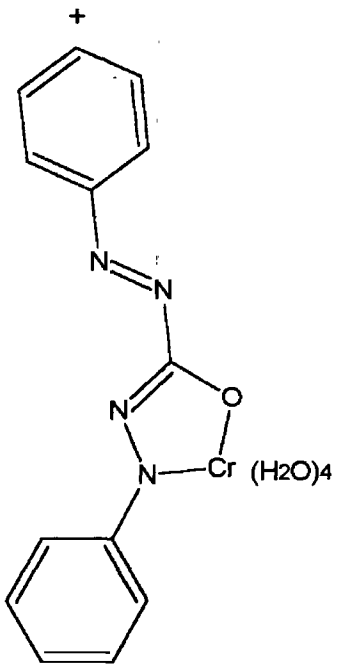


1,5-diphenylcarbazide (DPCI) 1,5-diphenylcarbazone (DPCO)

1,5-diphenylcarbazide does not complex directly with Cr^{6+}, but rather gets reduced to 1,5-diphenylcarbazone (DPCO) (a), along with the reduction of the hexavalent chromium to its trivalent form. It is only then that the trivalent chromium complexes with 1,5-diphenylcarbazone to form a chromium-1,5-diphenylcarbazonate (CrDPCO) (b).

The exact structure of the complex formed in (b) between Cr^{3+} and diphenylcarbazone is not yet clearly defined. However, the most probable compound resulting from this conjugation is a primary carbazonate complex (Figure 1.7).

Figure 1.7 Most probable developed formula of the chromium-carbazonate primary complex



1.4.1.1 UV/Vis investigation of the chromium-diphenylcarbazonate complex:

The complex formed from the conjugation of 1,5-diphenylcarbazide and chromium (VI) according to the reaction (a) and (b) is coloured (presence of chromophore as schematised on Figure 1.7). Therefore, this compound is typically quantitatively studied by ultra-violet visible spectrophotometry (UV/VIS). It has also been shown to be electroactive, and its electrochemical behaviour has been investigated [68-69, 71].

1.4.1.2 Materials and Method

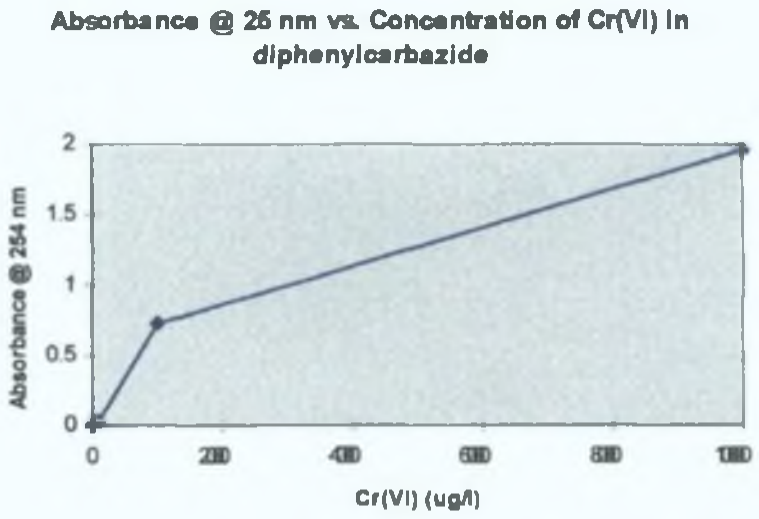
1,5-diphenylcarbazide (DPCI), sulphuric acid , and potassium dichromate were purchased from Sigma Aldrich. Diphenylcarbazide was purified by recrystallisation from ethanol absolute (Sigma-Aldrich).

15 ml of the water sample containing 0.01, 0.1, 1, 4, 10, 100, 1000 and 10000 ug.l^{-1} chromium(VI) were prepared and pipetted in 25 ml volumetric flasks. 1 ml of 3 mol.l^{-1} H_2SO_4 were pipetted and added to the flasks, and the contents were filled to the mark with a solution of 4 x 10^{-4} mol.l^{-1} DPCI (in 0.015 mol.l^{-1} H_2SO_4). The contents were left reacting for 5 minutes and the absorbance measured at 254 nm.

1.4.1.3 Results

From the results obtained, it was noticed that the greater the amount of hexavalent chromium present with a constant amount of 1,5-diphenylcarbazide, the greater the absorbance measured at 254 nm (Figure 1.8, Appendix 1.1).
This suggests the capability of 1,5-diphenilcarbazide to conjugate quantitatively with hexavalent chromium, to yield a coloured and hence measurable compound by ultra-violet and visible spectrophotometry.

Figure 1.8 Absorbance at 254 nm vs. concentration of Chromium (VI) in 1,5-diphenylcarbazide



1.4.2 Study of the chromium-diphenylcarbazonate complex by Cyclic Voltammetry

Chromium-1,5-diphenylcarbazonate can be adsorbed onto graphite, mainly due to the presence of its phenyl groups (these groups are often used in order to improve the adsorptivity of metal ligands onto graphite). This allows the complex to be pre-concentrated onto a graphite electrode, prior being analysed by stripping voltammetry.

Some researchers investigated the behaviour of 1,5-diphenylcarbazide, 1,5-diphenylcarbazone and the primary chromium carbazonate complex by voltammetry.
This project aimed at the investigation of the reaction between chromium and 1,5-diphenylcarbazide by electrochemistry, to determine the feasibility of the development of a chromium sensor based on this conjugation process, as well as on the electrochemical characteristics of such a conjugate.

Cathodic Stripping Cyclic Voltammetry of the Chromium-1,5-diphenylcarbazonate complex was carried out after a pre-concentration step. The reversibility of the conjugation reaction was also investigated [68, 69].

The purpose of this study was to get familiar with the instrument, as well as find out the optimal conditions for the determination of the chromium-diphenylcarbazonate complex.

1.4.2.1 Materials and Method

The polarographic instrument used was a Metrohm 693 VA-Processor, with 694 VA-Stand and 685 Dosimat. The system was composed of an epoxy-graphite, also called the 'ultra-trace graphite electrode' for working electrode, an Ag-AgCl 3 $mol.l^{-1}$ reference electrode, and a glassy carbon auxiliary electrode.

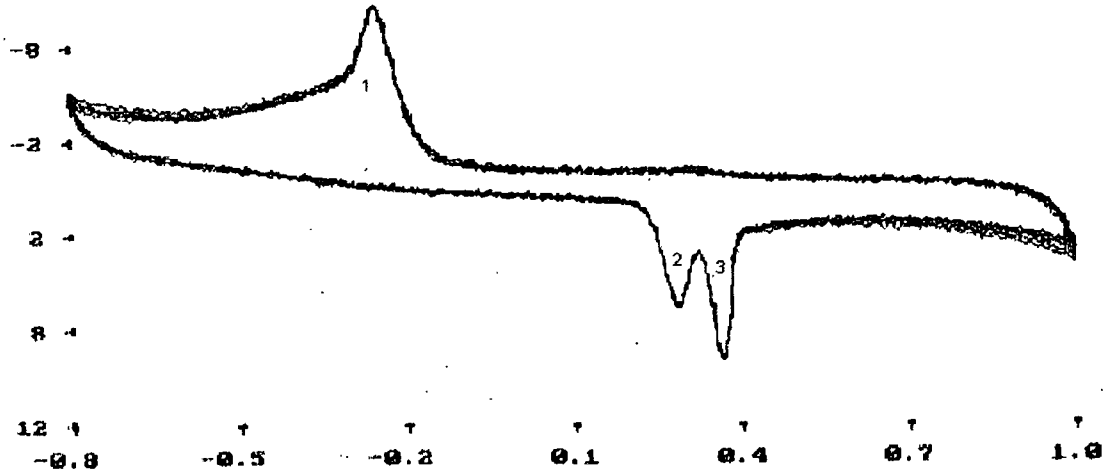
1,5-diphenylcarbazide (DPCI), sulphuric acid , and potassium dichromate were purchased from Sigma Aldrich. 1,5-diphenylcarbazide was purified by recrystallisation from ethanol absolute (Sigma-Aldrich).
A solution of 100 $\mu g.l^{-1}$ Cr and 4 x 10^{-4} $mole.l^{-1}$ in DPCI, in 1.5 x 10^{-2} $mol.l^{-1}$ H_2SO_4 was prepared.

Linear Sweep Voltammetry (DCT) was used at an epoxy-graphite ('Ultra-Trace', Metrohm, Switzerland). The deposition step was carried for 300 s at + 0.35 V vs. Ag-AgCl, at a rotational speed of 3000 rpm. The cyclic sweep was then carried out at a sweep rate of 40 $mV.s^{-1}$ from + 0.35 to +0.35 V vs. Ag.AgCl. The voltage step used during the sweeping was 5 mV, with a time step of 0.10 s. Each voltammogram was obtained after 2 preparation cycles and 5 measuring cycles .

1.4.2.2 Results

The cathodic-anodic-cathodic voltammogram of hexavalent chromium in 1,5-diphenylcarbazide can be observed in figure 1.9.

Figure 1.9 Cathodic-anodic-cathodic voltammogram of 100 $\mu g.l^{-1}$ Cr^{6+} and 4 x 10^{-4} $mole.l^{-1}$ DPCI in 1.5 x 10^{-2} $mole.l^{-1}$ H_2SO_4. Pre-concentration time : 300 s at + 0.35 V vs. Ag-AgCl. Initial voltage + 0.35 V, with a ramp up to + 1 V, down to - 0.8 V, and finally back to + 0.35 V.



Oxidation or reduction of the chromium-diphenylcarbazonate complex were obtained when the potential scan applied to the system went towards more positive (oxidation peaks 2) or negative (reduction peak 1) potential values referring to the + 0.35 V vs. Ag-AgCl adsorption potential. Previous research work attributed the oxidation peak 3 to the oxidation of un-reacted diphenylcarbazide. This peak was not observed when diphenylcarbazone was used as complexing agent.

The cyclic voltammograms obtained show the semi-reversibility of the reactions occurring between 1,5-diphenylcarbazide and Cr^{6+} (Figure 1.9).

Reduction Peak 1 - 0.256 V,

Oxidation Peak 2 + 0.283 V,

Oxidation Peak 3 + 0.363 V.

1.4.3 Determination of the Cr/DPCI complex by Cathodic Stripping analysis at an epoxy-graphite electrode

1.4.3.1 Materials and Method

The same instrument and reagents than in 1.4.2.1 were used in this study. The determination of the chromium-diphenylcarbazonate complex was carried out by linear sweep voltammetry at the epoxy-graphite rotating disc electrode (Metrohm, Switzerland), at a rotational speed of 3000 rpm.

A pre-concentration step was carried out at a voltage of + 0.35 V vs. Ag-AgCl for 300 s, followed by a linear stripping from + 0.35 V to - 0.5 V vs. Ag-AgCl, at sweep rate of 40 $mV.s^{-1}$.

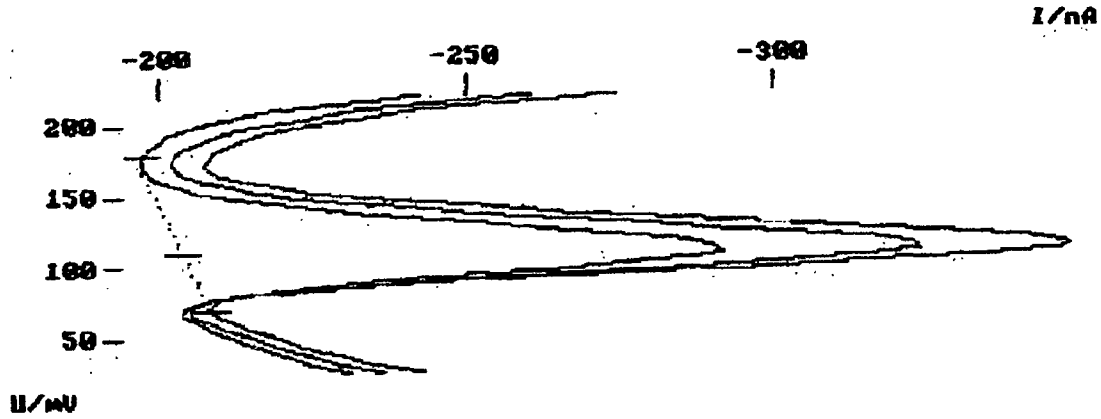
A 1 $g.l^{-1}$ Cr(VI) standard solution was prepared by dissolving 0.2830 g of potassium dichromate in 100 ml ultra-pure water.

A Cr-complex standard (1 ml = 0.5 $\mu g.l^{-1}$) was then obtained by pipetting 2.5 ml of 3.0 $mol.l^{-1}$ H_2SO_4 and 0.025 ml of the Cr(VI) standard solution into a 50 ml volumetric flask, which was then diluted to volume with 4 x 10^{-4} $mol.l^{-1}$ diphenylcarbazide (in 0.015 $mol.l^{-1}$ H_2SO_4).

15 ml of a 20 $\mu g.l^{-1}$ Cr^{6+} solution was pipetted into a 25 ml volumetric flask. 1 ml of 3 $mol.l^{-1}$ sulphuric acid was added to the flask, and 1.0 ml of the diphenylcarbazide solution. The solution was then brought up to volume with ultra-pure water, and left to stand for 15 minutes, and then analysed by standard addition.

1.4.3.2 Results

Figure 1.10 Voltammogram obtained for the direct current electrochemical determination of a solution containing 20 $\mu g.l^{-1}$ chromium by standard additions of 2 aliquots of 200 µl of 500 $\mu g.l^{-1}$ Cr/DPCI complex (200 ng). Deposition time : 300 s, 3000 rpm, at + 0.35 V vs. Ag-AgCl.



The results obtained after calibration were (Figure 1.10):

Cr concentration : 21.27 $\mu g.l^{-1}$,

Standard Deviation : 2.01 $\mu g.l^{-1}$ (9.43 %), 2 replica.

The main problems encountered during the study of the Chromium-diphenylcarbazonate complex was due to electrode fouling. The surface layer of the electrode needed to be removed frequently (usually every day) when sensitivity was observed to decrease. In addition, when the electrode was not used for a few days, the surface layer also needed to be removed before it could be reused successfully.

Surface regeneration of the electrode was obtained by using a ceramic cutter at a rotational speed of 600 rpm, yielding to a fresh and clean electrode surface for maximum adsorption of the complex (maximum surface area).

When the electrode was contaminated, the chromium-diphenylcarbazonate complex did not adsorb onto the epoxy-graphite, and no cathodic peak was observed at + 0.1 V vs. Ag-AgCl during the stripping.

The determination of chromium was successfully carried out down to trace levels (1 $\mu g.l^{-1}$) on the epoxy-graphite electrode.

Optimal adsorptive accumulation of the chromium/1,5-diphenylcarbazide complex was observed with a deposition time of 300 s rather than the preferred 60 s, as described in the method commercialised by Metrohm.

The main disadvantage of this method for the analysis of chromium is poor portability for on-site analysis.

The next step focused on the formation of the complex and its direct stripping at the electrode surface. The possibility of developing a carbon-paste or an epoxy-graphite electrode modified with 1,5-diphenylcarbazide was also investigated. Such an electrode may allow stripping and analysis on-site.

1.5 Development of a 1,5-diphenylcarbazide chemically-modified carbon-paste electrode

The possibility of determining chromium by using a 1,5-diphenylcarbazide chemically-modified carbon-paste electrode was investigated. In order to develop chemically-modified carbon-paste electrode, different methods of preparation were studied.

1.5.1 Modification of an epoxy-graphite electrode

1.5.1.1 Materials and Method

The reagents and chemicals used for the preparation of the chemically-modified carbon-paste electrode were graphite powder (1-2 micron, synthetic), 1,5-diphenylcarbazide (Aldrich), and dibuthylphtalate (Sigma).

Preparation 1

The procedure used to prepare the 1,5-Diphenylcarbazide chemically-modified carbon-paste was based on the article from Brainina and al. [68, 69]. In brief, 0.5 g of graphite powder was mixed with 1 ml of solution of 10^{-2} mole.l^{-1} 1,5-diphenylcarbazide in acetone and stirred until the acetone had completely evaporated. 0.15 ml of dibuthylphtalate was further added as a pasting liquid. This was stirred well to obtain a uniform paste.

Preparation 2

The procedure used to prepare this 1,5-diphenylcarbazide chemically-modified carbon-paste was based on the article from Paniaga and al. [34].

0.4 g of graphite powder was added to 17.5 ml of 1 % 1,5-diphenylcarbazide solution in 95 % ethanol (in water), in an agate mortar and stirred until the ethanol had evaporated. 400 µl of Nujol were added to the paste as a liquid binder.

Preparation 3

200 mg carbon powder and 100 mg 1,5-diphenylcarbazide were added to 120 mg of Nujol and thoroughly mixed in a mortar and pestle. This method was derived on the previous two methods.

The metrohm ultra-trace carbon-paste electrode was dipped into the carbon-paste obtained in the three cases, so that its tip was fully covered by a thin smear of the paste (< 1 mm, measured with a ruler). This smear was changed after every voltammogram to ensure electrode fouling did not affect the results obtained..

1.5.1.2 Results

A solution of 100 $\mu g.l^{-1}$ Cr^{+6} in 1.5 x 10^{-2} $mole.l^{-1}$ phosphate buffer was studied by cyclic voltammetry as previously described. However, no oxidation or reduction peak could be observed due to the magnitude of the background current vs. peak current obtained.

However, it could be seen that the chromium-diphenylcarbazonate complex was obtained in this case, due to the appearance of its characteristic pink colour, showing that the reaction occurred. The problem may have arisen from the fact that the complex could not adsorb onto the chemically-modified carbon-paste electrode or that the peak was hidden by the high background current. It could also be that the reaction only occurred from leakage of 1,5-diphenylcarbazide to the solution, or that the electrode was not electrically functional.

These observations were obtained for the three electrode types. No significant difference in the background current could be observed.

1.5.2 Preparation of a chemically-modified electrode based on 1,5-diphenylcarbazide

The development of a 1,5-diphenylcarbazide chemically-modified carbon-paste electrode was the next step of the project, which should be a cheap and a reusable electrode. This was carried out even though the previous step happened to be unsuccessful.

1.5.2.1 Materials and Method

The carbon-paste was prepared as previously mentioned (preparation 2), and packed in 1 ml surgical disposable syringes (insulin type). The electrical contact was made by using a silver wire at the centre of the electrode.

A solution of 100 $\mu g.l^{-1}$ in Cr^{6+} in 1.5 x 10^{-2} $mol.l^{-1}$ H_2SO_4 was prepared for testing. The Metrohm 693 VA-Processor and 694 VA-Stand were used, and the polarographic conditions were the same as previously stated in 1.4.2.1.

1.5.2.1 Results

High background currents were obtained so that no oxidation or reduction peaks were observable. A lower electrolyte (1.5 x 10^{-3} $mol.l^{-1}$ H_2SO_4) was tested, still giving high background currents, and not allowing any reduction or oxidation to be observed.

Large background currents may be caused by large surface area. In order to reduce this parameter, the paste was packed more tightly into the electrode body. Another attempt was carried out using a glass pasteur pipette as electrode body, the tip only being approximately 1 mm in diameter. However these alterations did not affect the background current in any way.

Different amounts of Nujol were used from 100 mg to 400 mg with 0.1 g of DPCI and 0.5 g of carbon powder, in order to increase the resistance of the electrode. However, this had no apparent effect on the intensity of the background current.

This constant background current was probably due to the lack of flexibility of the polarographic system used. The development of an electrode based on the chemical modification of a graphite-paste was therefore not investigated further.

1.6 Study of the use of over-oxidised polypyrrole as a chromium sensor

The aim of this part of the project was to try to determine whether over-oxidised polypyrrole could be used as a electrode material for the development of an hexavalent chromium sensor. It was carried out by coating a layer of polypyrrole at the surface of a gold electrode; the polymer film was then over-oxidised by scanning to anodic potentials.

1.6.1 Materials and Method

The instrument used in this part of the project was a metrohm 693 VA-Processor with a 694 VA-Stand. The working electrodes used were gold or glassy carbon rotating disc electrodes, a 3 $mol.l^{-1}$ Ag-AgCl reference electrode, and a platinum auxiliary electrode (Metrohm, Switzerland).

Freshly distilled pyrrole (Sigma), $NaNO_3$, KNO_3 (Sigma),
10^{-2} $mol.l^{-1}$ Sulphuric Acid (Sigma),
10^{-1} $mol.l^{-1}$ NaOH (Sigma).

Preparation 1

The procedure was based on the article from Wallace and al. [59] : polypyrrole was deposited onto a gold rotating disc electrode at + 0.8 V vs. Ag-AgCl (DCT Mode) at a rotational speed of 2000 rpm for 2 min, from a solution of 10^{-1} $mol.l^{-1}$ pyrrole and 10^{-1} $mol.l^{-1}$ $NaNO_3$. The fresh layer of polypyrrole was then over-oxidised in 10^{-2} $mol.l^{-1}$ H_2SO_4 by scanning potential from - 0.4 to + 1.3 V vs. Ag-AgCl for 30 min.

Preparation 2

A polypyrrole film was deposited onto a glassy carbon rotating disc electrode from a fresh solution of 10^{-1} mol.l^{-1} pyrrole in 10^{-1} mol.l^{-1} potassium nitrate at a potential of + 0.8 V vs. Ag-AgCl [61]. The film was then over-oxidised in aqueous 10^{-1} mol l^{-1} sodium hydroxide by cyclic voltammetry between 0 and + 1.2 V vs. Ag-AgCl.

The freshly polymerised films were investigated as potential chromium sensors using a 100 µg.l^{-1} Cr^{6+} solution. Pre-concentration was carried out for 180 s at a potential of + 0.8 V vs. Ag-AgCl in both cases to the modified electrode (2000 rpm) in order to amplify the stripping of the Cr^{6+} response.

A pre-concentration step was carried out by direct current mode, with a voltage step of 0.5 s, a scan rate of 10 mV.s^{-1}, a modulation amplitude of 100 mV, a pulse width of 50 ms, and a rest time of 10 s.

The stripping of the Cr^{6+} was carried out by differential pulse voltammetry, with a step width of 0.5 s, a scan rate of 10 mV.s^{-1}, a modulation amplitude of 100 mV, a pulse width of 50 ms, a time measurement of 20 ms. The sweep rate was 12 mV.s^{-1} and the potential step was 6 mV.

1.6.2 Results

Background noise appeared to be an omnipresent and major problem during the stripping of the Cr^{6+} solution, hiding its response, assuming a response existed.

Lowering the strength of the electrolyte did not have any noticeable effect on the background current. As a result, the freshly prepared polypyrrole coated electrode was not valid as chromium sensor.

1.7 Study of the determination of trivalent chromium by adsorptive differential pulse voltammetry (AdSV)

A different method of determination of chromium by adsorptive pulse voltammetry (AdSV) was studied. In this case, the method was not sensitive to hexavalent but trivalent chromium.

The determination of trivalent chromium by differential pulse at a dropping mercury electrode (DME) was investigated as per the article from Golimowski and al [28].

1.7.1 Materials and Method

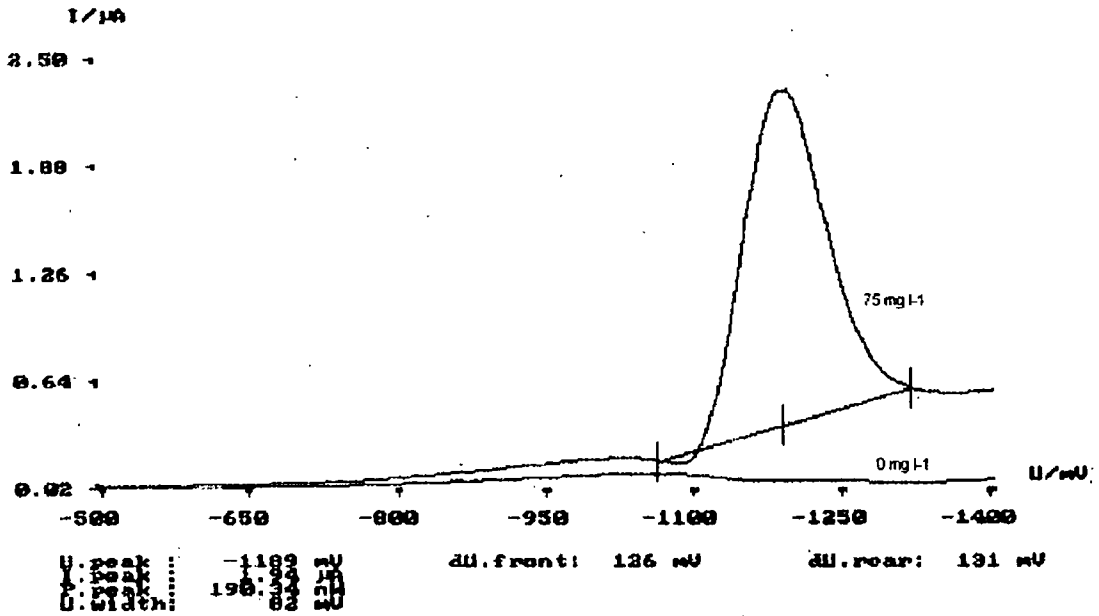
Solutions of 0, 1, 10, 20, 75 and 100 mg.l^{-1} Cr^{3+} were prepared in 10^{-3} mol.l^{-1} EDTA, 0.5 mol.l^{-1} $NaNO_3$ and 5 x 10^{-2} mol.l^{-1} CH_3COONa in ultra-pure water (all reagents from Sigma-Aldrich).

The adsorptive stripping voltammetry method was carried out with a pulse amplitude of 50 mV, a time measurement of 5 ms, a time step of 0.3 s, and a pulse of 40 ms. The stripping step started from an initial voltage of - 0.5 V, to a final voltage of - 1.5 V vs. Ag-AgCl, at a weep rate of 50 mV.s^{-1} and a voltage step of 6 mV.

1.7.2 Results

The voltammograms obtained for a blank solution and a solution containing 75 mg.l^{-1} hexavalent chromium in EDTA, sodium nitrate and sodium acetate, by adsorptive differential pulse voltammetry at a dropping mercury electrode. The results obtained experimentally (Figure 1.11, Appendix 1.2) shows the oxidation potential obtained for Cr^{3+} in a solution containing EDTA and sodium Nitrate at - 1.19 V vs. Ag-AgCl, and a peak intensity of 1.94 µA.

Figure 1.11 Voltammograms obtained for solutions of 0 and 75 $mg.l^{-1}$ hexavalent chromium in a solution containing 10^{-3} $mol.l^{-1}$ EDTA, 0.5 $mol.l^{-1}$ $NaNO_3$ and 5 x 10^{-2} $mol.l^{-1}$ CH_3COONa.



A set of solutions was therefore prepared and analysed following the same method to obtain a series of voltammograms (Figure 1.12) and a calibration curve (Figure 1.13) between 1 and 100 $mg.l^{-1}$ Cr^{3+}.

A linear calibration was obtained, and a correlation obtained between 1 to 100 $mg.l^{-1}$ with a sensitivity of 0.0296 $mg^{-1}.l.mA^{-1}$ and an intercept of 0.0365. The correlation factor was 0.9941.

Figure 1.11 Linear calibration of solutions of 1, 10, 20, 75 and 100 mg.l^{-1} Cr^{3+} in 10^{-3} mol.l^{-1} EDTA, 0.5 mol.l^{-1} $NaNO_3$ and 5 x 10^{-2} mol.l^{-1} CH_3COONa, by adsorptive differential pulse voltammetry at a dropping mercury electrode :

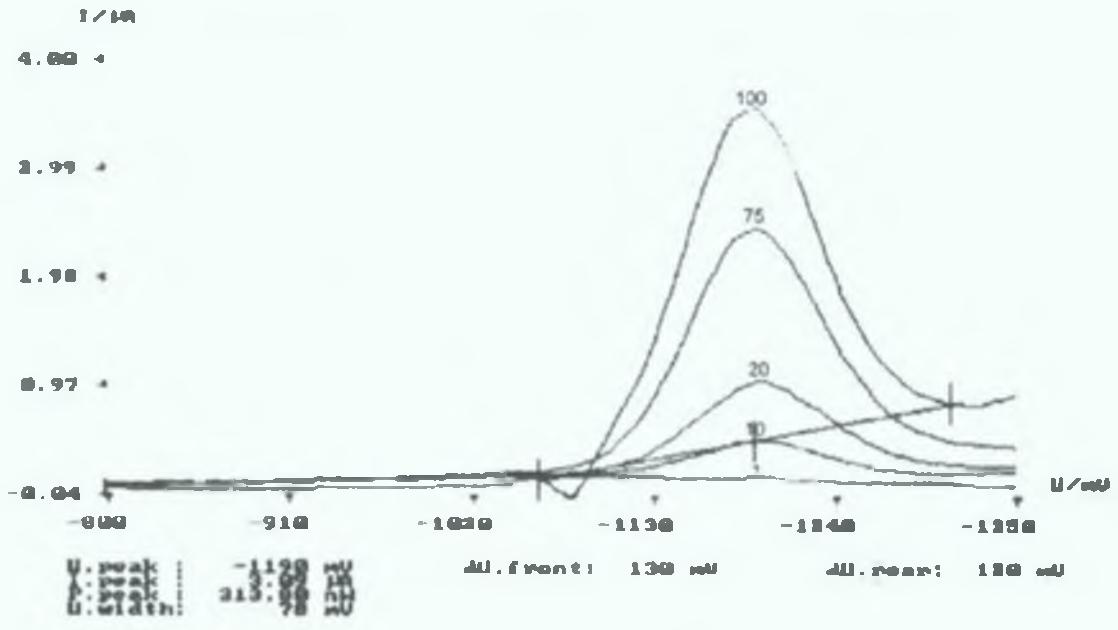
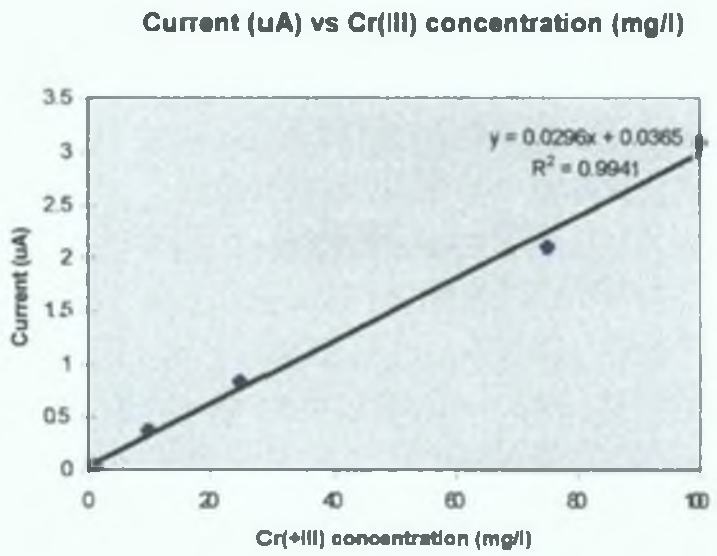


Figure 1.12 Calibration curve for 1, 10, 20, 75 and 100 mg.l^{-1} Cr^{3+} in a solution 10^{-3} mol.l^{-1} EDTA, 0.5 mol.l^{-1} $NaNO_3$ and 5 x 10^{-2} mol.l^{-1} CH_3COONa.



1.8 Discussion

This part of the project was aimed principally at the investigation and study on the development of sensors for the analysis of heavy metals. An extended literature study was carried out which covered the definition of sensors (physical, chemical and biological), and their main applications for heavy metals monitoring. It also covered the definitions of stripping voltammetry, polarography, and defined the most widely used working and reference electrodes, as well as the main electroanalytical techniques used for heavy metals determination.

This part of the project was principally a theoretical and experimental familiarisation of the techniques and electrochemical principles involved in the use of solid electrodes. The determination of chromium in water was studied by using different solid state electrodes such as epoxy-graphite, glassy-carbon, or mercury electrodes.

Qualitative and quantitative investigation of the chromium-diphenylcarbazide complex was also obtained by ultraviolet and visible spectrophotometry.

The main problems encountered during the electrochemical study were centred around the highly rigid and non-user-friendly aspects of the metrohm polarograph.

The procedure for the determination of chromium by adsorptive stripping carried out on the epoxy-graphite 'Ultra-Trace' electrode developed by metrohm was successfully carried out. Such electrode was specially developed for the instrument for this analysis, based on the articles studied in 1.4, and therefore met particular specifications, which could not always be met by other electrodes designed in the lab.

Samples containing chromium concentrations as low as 1 $ug.l^{-1}$ can successfully be analysed using this particular method.

The cyclic voltammetric behaviour of the complex formed between 1,5-diphenylcarbazide and chromium was also investigated at the epoxy-graphite electrode. One reduction peak was observed, whereas two oxidation peaks were present. The results obtained indicates the semi-reversibility of the reaction as well as the absorption of 1,5-diphenylcarbazide to the electrode [67].

Other modified electrodes developed during this experimental study showed high background currents or could not be recognised by the instrument used. More versatile potentiostats are presently on the market (some of them being portable for on-site analysis), and would allow more flexibility which would facilitate research.

Using the 1,5-diphenylcarbazide chemically-modified carbon-paste electrodes developed at the laboratory, the reaction between the chromium ions and 1,5-diphenylcarbazide was obtained (i.e. presence of the coloured complex), but the stripping current was covered up by high background current. Different chromium concentrations were investigated, but the high background noise did not allow any successful analysis.

It was therefore impossible to determine whether the complex obtained was effectively adsorbing at the electrode surface, and if it was stripped. Different types of carbon-pastes were tested (3 types), as well as the area of the electrode surface, but no electrochemical change could be observed.

Future work which could be undertaken on this part of the project should focus on the manufacture of wax based electrodes (i.e. candle wax, modifier and graphite embedded), as this type of electrode possesses characteristics such as low background current, uniform dispersion of the modifier, and the possibility of surface regeneration by scrapping. Besides, the ultra-trace electrode developed by Metrohm for the determination of chromium using 1,5-diphenylcarbazide is based on this type of material, and suggest more suitable characteristics than carbon-paste.

The investigation of the complexation reaction between hexavalent chromium and 1,5-diphenylcarbazide was carried out in depth at various solid and mercury electrodes. From the results obtained in the project, it can be said that future work could focus on the development of disposable screen-printed carbon-paste electrodes, modified with 1,5-diphenylcarbazide.Such devices may allow on site electrochemical analysis and speciation of Cr^{6+} over Cr^{3+}.

The monitoring of trivalent chromium could be also investigated by developing similar sensors modified with 1,5-diphenylcarbazone.

However, most interest should be given to 1,5-diphenylcarbazide chemically-modified carbon-paste screen-printed sensors, which would allow the determination of the hexavalent chromium, which has the greater adverse environmental impact.

CHAPTER 2.0

USE OF GLUTATHIONE s-TRANSFERASE FOR THE DEVELOPMENT OF AN ORGANOCHLORINE FLOW-THROUGH SENSING SYSTEM

2.1 Introduction :

2.1.1 Sensing organochlorine and organic compounds ?

Environmental pollution in the last century

As stated in 1.3, human activities and industrial revolution have modified natural cycles, causing regional and global redistribution of more or less hazardous elements in aquatic and terrestrial systems, up to critical concentrations in various cases. Human history has never seen a greater chemical load placed on the environment as there is today, and the chemical industry continues to generate an ever-increasing amount of pollutants. It is estimated that between 50,000 and 100,000 chemicals are now commonly used world-wide (Table 2.1). Substantial evidence has arisen about the contamination of the environment by waterways, soil and air, as well as our bodies, with toxic chemicals.

Table 2.1 Production data for the UNEP Persistent Organic Pollutants (POPs) pesticides [72]

Organochlorine Pesticides	Initial Producer	Year of Introduction	Global Use (tonnes)
Aldrin/Dieldrin	Shell	1948-50	> 130,000
Endrin	Velsicol and Shell	.	> 2,300
Chlordane	Velsicol	1947	3,000,000
DDT	Ciba-Geigy AG	1946	30,000*
HCB	.	1945	> 100,000
Heptachlor	Velsicol	1952	N/A
Mirex	Allied Chemical Co.	1946	N/A
	Hooker Chemical	1958-59	> 1,500
Toxaphene	Hercules Powder Co.	1946	1,333,000

* Production estimated (WWF 1998) and believed to be taking place in India and China.

Pollution by organochlorine compounds is an important environmental and health feature, and an extensive number of these organic compounds are listed in the 76/464/EEC Directive, also known as the 'Dangerous Substance Directive' [73].

Use of organochlorine compounds as pest control

Huge amounts of organochlorine compounds are used as pesticides, as well as in chemical, pharmaceutical and halide industries. Many of these organochlorine compounds possess carcinogenic and/or teratogenic characteristics, and are therefore a threat to the public health and to the quality of ground, river and lake water. The organochlorine pesticides were introduced in Australia in the mid 40's, in different forms (wettable powders, liquids), and include DDT, dieldrin, heptachlor and chlordane.

Many of these pesticides are listed within the 'List of 129 compounds' and in the UNEP POPs, due to :

i) their resistance towards chemical, physical and biological means of degradation, their half-lives ranging from months to years, to decades in some cases. Aldrin, dieldrin, endrin, chlordane, HCB, heptachlor, mirex, toxaphene are classified as Persistent Organic Pollutants (POP's). POP's are defined as compounds that will find their way into the environment, even many years after production may have ceased (i.e. pesticides, industrial chemicals, and unattended by-products of combustion and industrial processes);

ii) their toxicity to human and other animals, and most aquatic life, and their short-term or long-term impacts at low concentrations; and

iii) their bio-accumulation in the fatty tissues of plants and animals. This means that animals higher in the food chain such as birds of prey or humans accumulate higher levels of the pesticides than animals lower down the food chain.

It is therefore essential to obtain a fast and sensitive way to determine this type of compound. On the other hand, *in situ* determination appears primordial in the case of effluent monitoring, as most organochlorine pollutants are produced in the chemical, mill, pulp and paper industries, sludge incinerators, oil refining (used as catalysts) or steel and iron industries, where fast action may be required in the case of release of high pollutant concentrations.

A good indication of the persistence of compounds in the environment is their absence from remote areas such as the Arctic or deep oceans. Many POPs were identified in the Arctic (Table 2.2) by the Arctic Monitoring and Assessment Programme (AMAP, 1998), which is of concern because of their global distribution via air and water currents [74-76].

Table 2.2 Some of the world leading companies involved in the manufacture of one or more POPs contaminating the Arctic [77]

Hoescht (Germany)	Heptachlor, HCH, Endosulfan
BASF (Germany)	Aldrin, Lindane, Methoxychlor
Bayer (Germany)	Hexachlorbenzene, PCB's Endosulfan, Lindane
ICI (UK)	DDT, Aldrin, Endosulfan, Lindane
Rhone-Poulenc (France)	Aldrin, Endosulfan, Lindane, Methoxychlor, PCP
Du Pont (US)	Endosulfan
Shell (Switzerland/UK)	Aldrin, Dieldrin, HCH, Endosulfan
Akzo Nobel (Netherlands, Sweden)	Endosulfan, Lindane, PCP, Methoxychlor
Sandoz (Switzerland)	Chlordane, Endosulfan, Lindane, Aldrin
Monsanto (US)	PCBs
EniChem (Italy)	DDT

Drinking water quality

The Drinking Water Directive 80/778/EEC [78] was transposed in Ireland in 1988 by the publication of the European Communities (Quality of Water Intended for Human Consumption) Regulations 1988. This sets out Guide Values and Maximum Admissible Concentrations (MAC, also called the National Limit Value in Ireland, Figure 2.3) for 60 compounds. The Directive states that national standards must conform to the MAC as minimum standards, Guide Values being standards to work towards in the long term (Table 2.3).

Table 2.3 EC guide, EC MAC and Irish NV values for pesticides, related products and organochlorine compounds [78].

Compounds	EC Guide Values ($\mu g.l^{-1}$)	EC MAC ($\mu g.l^{-1}$)	Irish NV ($\mu g.l^{-1}$)
Pesticides and related products			
i) individual substances	N/A	0.1	0.1
ii) total	N/A	0.5	0.5
Organochlorine (other than pesticides)	1	N/A	100

The Environmental Protection Agency (EPA) set up similar standards on the concentration of pesticides in water intended for human consumption at 0.1 $\mu g.l^{-1}$, with a Maximum Admissible Limit (MAC) at 1 $\mu g.l^{-1}$.

The World Health Organisation also described standards for drinking water values for chemicals of significance to health (1993). Many organochlorine pesticides appear in the list, including Aldrin/Dieldrin (0.03 $\mu g.l^{-1}$), DDT (2 $\mu g.l^{-1}$), Heptachlor (0.03 $\mu g.l^{-1}$), Lindane (2 $\mu g.l^{-1}$). However, these values are only described as Guide Values [79].

Instrumental analysis of organochlorine

The common instrumental analyses for the determination of such pollutants include Gas-Chromatography Mass Spectrometry (GC-MS) with Electron-Capture Detector (GC-ECD) [80-87], often after pre-concentration by micro-extraction [88], High-Performance Liquid-Chromatography (HP-LC) and/or Liquid-Chromatography Mass-Spectrometry (LC-MS) [89]. Thin Layer Chromatography (TLC) is also commonly used to separate insecticides [90].

Best results are obtained by Gas Chromatography Mass Spectrometry and by Gas Chromatography with Electron-Capture Detector. As a result, most of the determination of pesticides in water or other environmental samples is currently carried out by GC-MS and GC-NPD as described in the EPA method N° 525.

GC-MS is a quick, sensitive instrumental techniques, that allows the determination of up to 60 analytes in the same run, and possesses a wide working linear range and can be automated. GC-ECD is even more sensitive, but the qualitative advantage of mass spectral analysis means that GC-MS is used in preference to GC-ECD [83-85, 87].

LC-MS shows has more an utility for the investigation of the fate of the pesticides as well as their metabolites [89], to understand better their characteristics in the environment, than for pure quantitation purposes.

However, the main drawbacks to such determination practices arise, such as the time and money consuming sample preparation, and lack of portability. These chromatographic instruments are also expensive to purchase and require skilled technicians, and they are highly dependant on the quality of the sampling stage. As a result, Enzyme-Linked Immunoassays (ELISA) were developed for the determination of pesticides [86, 90-91] or other organochlorine compounds *in situ*.

2.2 Enzyme Characteristics and Kinetics

2.2.1 Activation Energy and Transition State - Role of an Enzyme

Enzyme kinetics are a specific branch of chemical kinetics, which focus on the kinetic theory of matter. This theory states that a solution can be viewed as a group of molecules moving at particular velocities. During their movement, some will collide with each other, each collision involving a certain amount of energy. If this energy is sufficient, the molecules combine to form a metastable intermediate (also referred as being in the transition state, E-S*). The activation energy is defined as being the energy required to form this intermediate to be formed. However, if the energy involved during the collision is insufficient, the molecules only bounce off each other.

An enzyme can be defined as a biological catalyst which accelerates a specific chemical reaction, whilst remaining unchanged at the end of the reaction. An enzyme catalyses the reaction by lowering the activation energy required for the reaction to occur. The reacting molecules can therefore reach the transition state at which they will combine with the reactants more easily, the transition state being lower than for the un-catalysed reaction.

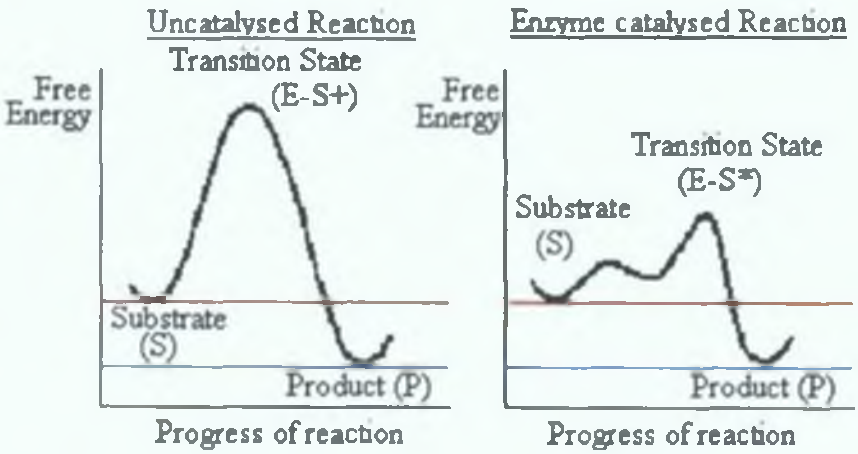
Enzymes are proteins found in all types of cells. A cell will contain hundred of enzymes depending on its function. An enzyme is 3D-shaped, formed by the highly complex and precise folding of a single polypeptide chain (the typical molecular weight is between 13,000 and 50,000 Atomic Mass Units) [93]. A small portion of this molecule, typically defined by 5 to 10 amino acids spatially arranged in a specific relative conformation, will form the active site of the enzyme, also known as the catalytic site.

In order to prevent any non-specific interactions and access to potential inhibitors, as well as to promote the specificity towards the substrate(s), the active site is situated at the interior of molecule.

This active site is designed in such a way that it will help the redistribution of the electron density within the substrate molecule by creating ionic and hydrogen bonds. These interactions are then responsible for the lowering of the intermediate transition state of the reaction.

The function of this enzyme is to lower the activation energy required by the reacting molecules to reach the transition state at which they will combine with the reactants to form a different transition state (E-S*) from that involved in the un-catalysed reaction (Figure 2.1).

Figure 2.1 Effects of an enzyme on free energy, transition state and progress of a reaction.



The study of enzyme kinetics commenced in the early 1900's by the derivation of the Michaelis-Menten Equation, which describes the relationship between the substrate concentration and the rate for the reaction :

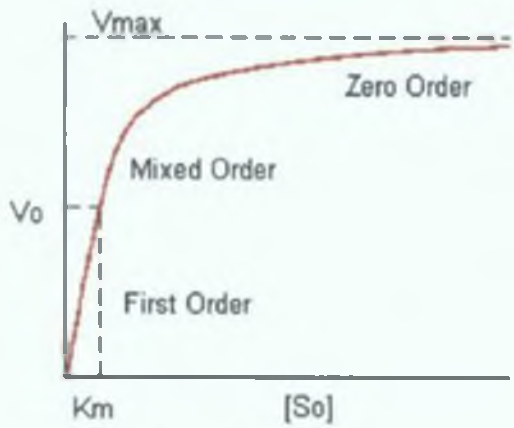
$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} E\text{-}S \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} E + P$$

In an enzyme - catalysed reaction, the enzyme (E) combines with a substrate (S) to form an Enzyme-Substrate (E-S) complex that will then breakdown to form a product (P) and the starting enzyme (E). K_1, K_{-1}, K_2 and K_{-2} are the reaction rate of formation and breakdown of the complex.

At low substrate concentrations [S], an enzyme reaction follows first order kinetics with respect to the substrate concentration (i.e. the rate of formation of the product is dependant on [S]). However, at high [S] value, the substrate concentration has no longer an effect on the reaction rate, and the reaction therefore follows zero order kinetics. As a result, when the concentration of the substrate is not high enough to fully saturate the enzyme present in the reaction, and the reaction is not first order.

A plot of initial substrate concentration [S_o] vs. the Initial Velocity V_o can be obtained for an enzyme-catalysed reaction, gives a rectangular hyperbola (Figure 2.2) :

Figure 2.2 Initial substrate concentration vs. Initial Velocity for enzyme-catalysed reactions



When the concentration of the product P is very small (i.e. at early stage of the reaction), K_2 is insignificant and can therefore be neglected.

The Michaelis-Menten assumption states that the equilibrium between the concentration of the enzyme and the substrate, and the complex occurs almost instantaneously, and is maintained as the rate of breakdown of the complex to enzyme and products is too slow to disturb this equilibrium.

The rate of formation of the complex at any time t is equal to its rate of breakdown :

$$K_1.[E].[S] = K_{-1}.[E\text{-}S]$$

The value of the dissociation constant K_s of the complex E-S can now be expressed :

$$\frac{[E].[S]}{[E\text{-}S]} = \frac{K_{-1}}{K_1} = K_s$$

If $[E_0]$ is defined as the total enzyme concentration :

$[E_0] = [E] + [E\text{-}S]$

$[E] = [E_0] - [E\text{-}S]$ can be placed into the expression of Ks, to yield to

$$[E\text{-}S] = \frac{[E_0].[S]}{[S] + [K_s]}$$

The concentration of the complex [E-S] governs the rate of formation of the product according to the following relationship :

$$V_0 = K_2.[E\text{-}S]$$

The Michaelis-Menten equation can therefore be expressed, expressing V_0 as a function of K_2, $[E_0]$, [S], and K_S.

The limiting initial velocity V_{max} is reached at high substrate concentrations :

$$V_{max} = K_2.[E_0]$$

A second expression of the Michaelis-Menten equation can be determined, this time expressing V_0 as a function of V_{max}, [S], and K_S :

$$V_0 = \frac{V_{max}.[S]}{[S] + [K_s]}$$

2.2.2 Enzyme Denaturation

Denaturation of an enzyme is defined as the irreversible disruption or perturbation of the secondary, tertiary or quaternary protein structure of the active site. Even small conformational changes in these key amino-acids will change irreversibly the enzyme activity and specificity. Denaturation may be due to changes in pH, temperature, pressure, time, exposition to ultra-violet, organic solvents or chemicals. Many parameters need to be observed and carefully taken into account when studying or using enzymes, which constitutes a main problem for analytical work. As a result, it is obvious that tests should be carried out on a frequent basis in order to prevent or to detect denaturation.

2.2.3 Enzyme Immobilisation :

The enzyme immobilisation is defined as its confinement, without destroying its active site, at a particular surface.

Enzyme immobilisation can be either *chemical* (which requires the formation of a covalent bond between two protein molecules or between the protein and the support material) or *physical* (which attaches the enzyme by physical forces such as Van der Waals forces, hydrogen bonding, entrapment or containment).

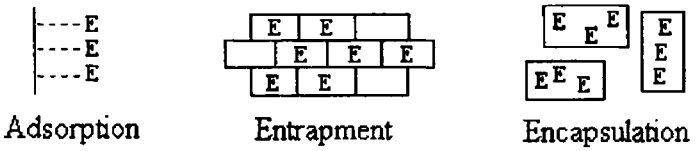
The different physical immobilisation techniques can be observed in Figure 2.3 :

i) Adsorption can be done by binding through hydrogen bonds, Van der Waals forces, multiple salts linkages, or through the formation of electron transition complexes. Physical conditions such as pH, temperature, ionic strength, enzyme concentration and solvent used will affect the strength of the attachment;

ii) Entrapment involves the enzyme being entrapped into a water-insoluble polymer by adding the enzyme to a monomer, which is then polymerised; and

iii) Encapsulation involves the enclosure of the enzyme within spherical and semi-permeable polymer membranes (1 -100 μm diameter).

Figure 2.3 Physical immobilisation of enzymes : adsorption, entrapment and encapsulation.



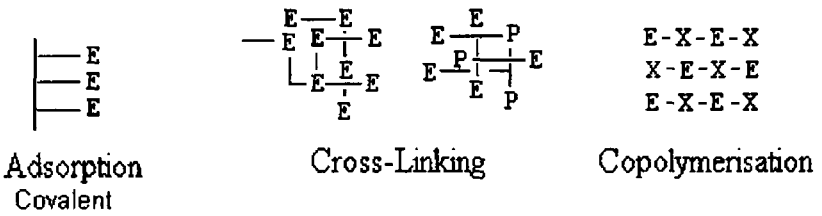
Chemical immobilisation techniques are also widely used (Figure 2.4) :

i) Covalent immobilisation to a functionalised insoluble support can be obtained by using material such as controlled porous glass (CPG), cellulose membrane, nylon membrane and carbon modified with reagents such as carbodiimide, glutaraldehyde, and cyanogen bromide;

ii) Cross-linking immobilisation is usually employed to give functionality onto a solid support, or to create proteins linkages. Glutaraldehyde is the most employed cross-linking agent; and

. *Co-polymerisation* occurs as the covalent incorporation of the enzyme into the polymer support.

Figure 2.4 Chemical immobilisation of an enzyme : adsorption, cross-linking and co-polymerisation.



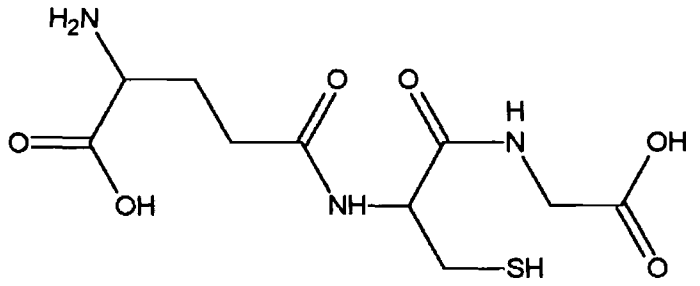
Where E, P and X are the enzyme molecule, the protein molecule (typically BSA) and the monomer molecule respectively [92].

2.3 Reduced Glutathione and Glutathione s-Transferase

Plants and other organisms possess mechanisms to defend themselves against herbivores, insects, pathogens as well as xenobiotics (foreign chemical compounds) that are harmful to their survival. This is done in two distinct strategies called *constitutive* (natural or innate) and *induced* (created by an external agent) defences.

Glutathione (GSH, Figure 2.5) is a tripeptide composed of residues of glycine (Gly), cysteine (Cys) and glutamic acid (Glu). Important known roles of this molecule range from the protection of red cells from oxidative damage to the maintenance of Haemoglobin in the ferrous state [94].

Figure 2.5 γ-L-glutamyl-L-cysteinylglycine (glutathione)



Many studies have been carried out on the determination of glutathione by polarography [95].

Cathodic stripping voltammetry at dropping mercury electrodes (DME), hanging mercury electrodes (HMDE) [96], gold / mercury amalgam micro-electrodes [97], glassy carbon rotating disc electrodes or chemically-modified carbon electrodes [98]. Flow-injection amperometry at an ruthenium-containing film modified glassy carbon electrode was also investigated by Cox and al. [99]. The determination of glutathione by flow-injection analysis (FIA) based on immobilised enzyme columns was also the focus of numerous studies [100].

High-performance liquid chromatography was also performed, the detection carried out at a prussian blue film-modified electrode [101].
A certain number of screen-printed electrodes modified with electron mediators (ferrocene and some of its derivatives) were also studied [102].

Many pesticides have different metabolic pathways, many use GSH/GsT. The major three metabolic pathway degradation reactions of pesticides in plants are N-Dealkylation with glutathione [103-104]. This latter is done in conjunction with glutathione and glutathione s-transferase (GsT). In this case, glutathione forms a conjugate with the intruding foreign compound carrying an electrophilic centre, which reaction is catalysed by GsT [105].

Glutathione s-transferases are a family of enzyme responsible responsible for catalysing the conjugation of GSH to certain xenobiotic, by covalently linking to reduced glutathione to a hydrophobic substrate, thus forming a less reactive, more polar and more water-soluble glutathione s-conjugate.

$$R\text{-}X + GsH \xrightarrow{GsT} R\text{-}SG + HX$$

Glutathione s-transferases are a family of isoenzymes consisting of glutathione s-alkyltransferase, s-aryltransferase, s-aralkyltransferase, s-alkenetransferase, s-epoxidetransferase, r-transferase. The different isoenzymes catalyse the conjugation of different chemicals to GSH, i.e. different xenobiotic compounds react with GSH.

However, the rates of enzyme catalysed reactions vary greatly as different substrates have different affinities for the enzyme. This type of enzymes are present in most organisms, including plants, animals, protozoa, fungi, and bacteria. The increase in glutathione and GsT have been linked to organism resistance to a variety of physical and chemical stresses encountered in the environment [106].

Most commonly studied substrates of GsT are 1,2-dichloro-4-nitrobenzene, 1,2-epoxy-(n-nitrophenoxy) propane, ethacrynic acid, p-nitrophenethyl bromide, p-nitrobenzyl chloride, bromosulfophtalein.

Glutathione s-aryl transferase combines with a substrate so that a hydrogen halide is eliminated (e.g. dichloronitrobenzene, chlorodinitrobenzene). Important degradation reactions catalysed by this type of enzyme are linked with pesticides such as atrazine and some other chlorinated triazine derivatives in plants.

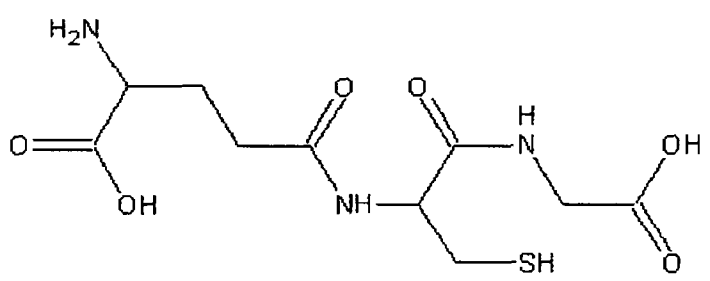
In contrast, glutathione s-alkyl transferase catalyses reactions in which glutathione attaches to what was the smallest part of the original molecule. It will however react with the alkyl halides in a similar manner to the previous group of transferase. This reaction is particularly interesting for methyl removal in organo-phosphorous pesticides such as methyl parathion, mevinphos or dichlorvos.

Glutathione s-epoxide transferase catalyses reactions in which an epoxide ring is opened to enable the attachment of glutathione. This type of enzyme is particularly important in the pesticide pathway of carcinogenic compounds such as dieldrin, endrin, heptachlor epoxide as well as all organochlorine epoxides [107].

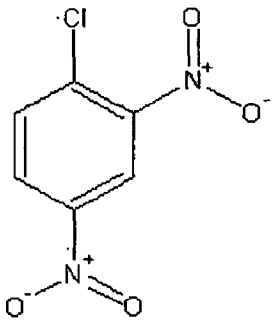
Other related enzymes are the glutathione peroxidases (enzyme whic catalyses the degradation of hydrogen and organic peroxides), glutathione synthesise (which catalyses the activation of the carboxyl group of cysteine by ATP) and glutathione reductase (catalyses the reduction of the oxidised glutathione to the reduced glutathione).

The conjugation reaction of GSH with 1-chloro-2,4-dinitrobenzene (Figure 2.6) occurs more rapidly than any other organochlorine compound, and has been thoroughly studied. This substrate is most commonly studied as its speed of the reaction with GSH is faster relative to other substrate.

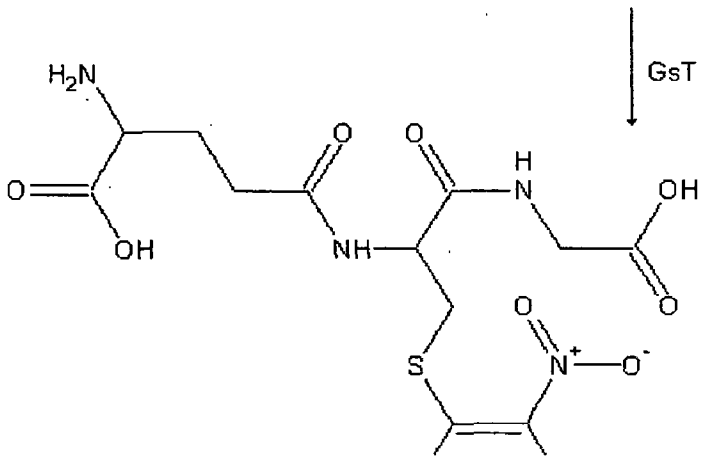
Figure 2.6 Reaction catalysed by GsT between 1-chloro2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH)



reduced glutathione (GSH)



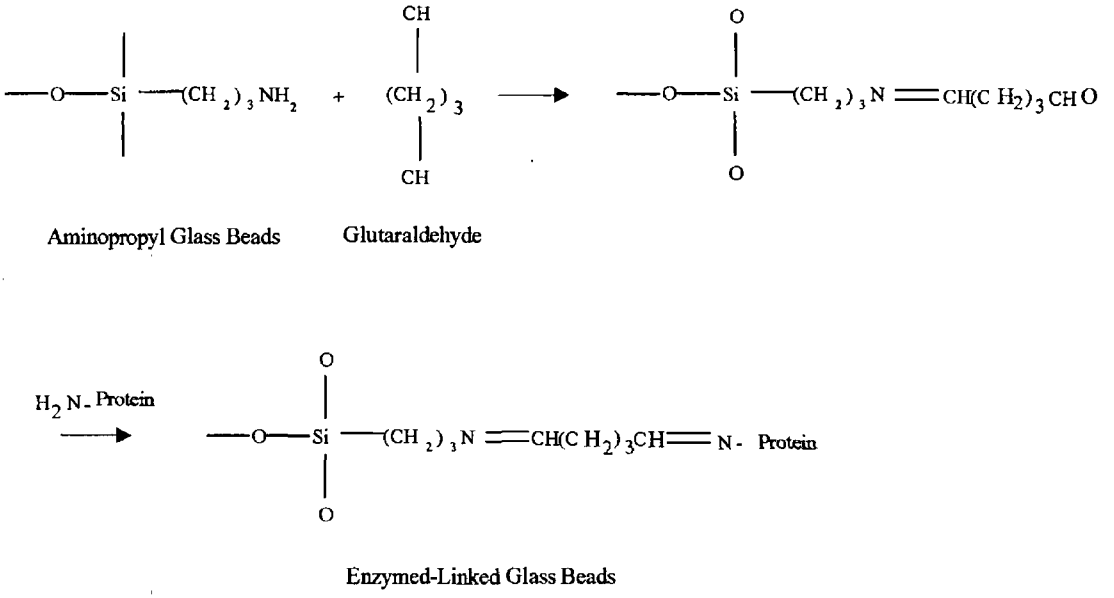
1-chloro-2,4-dinitrobenzene



Glutathione s-transferase can be immobilised onto aminopropyl porous glass beads (Figure 2.7), and packed into a reaction column. This allows for the reuse, and in certain case continuous use of the enzyme.

The first step involves the activation of the aminopropyl glass beads by reaction with glutaraldehyde (Figure 2.7), whereas the second step involves adding the enzyme (GsT) to the activated beads (Figure 2.8). The final step is then the coverage (or 'capping') of any un-reacted and available site with a neutral enzyme such as bovine serum albumin.

Figure 2.7 Protein immobilisation onto aminopropyl porous glass beads



The reaction between CDNB and GSH is catalysed by the GsT immobilised on the glass beads. The main interest in immobilising the enzyme onto aminopropyl porous glass beads is the acceleration of the conjugation of GSH with CDNB. As all thiol, GSH is an electrochemically active compound and this electrochemical activity may be altered upon complexation to the CDNB molecule.

2.4 Experimental study of reduced glutathione by polarography and voltammetry

2.4.1 Introduction

Reduced glutathione has been widely studied at mercury electrodes, GSH forming an amalgam with mercury. The following work focused on the investigation of the electroanalytical analysis of GSH at a dropping mercury electrode by square-wave voltammetry, by varying parameters such as pH, ionic strength, concentration of buffer, time step, frequency of signal, time measurement.
This was carried out to understand the behaviour of this organic compound and maximise the detection limits of its analysis by polarography and voltammetry.

A calibration curve and a dynamic range were obtained at the dropping mercury electrode, by square-wave voltammetry at a dropping mercury electrode.

Reduced glutathione will then be investigated by cyclic voltammetry at various other electrode types to determine their relative effectiveness, in view of a future use in a flow-cell.

2.4.2 Study of the polarographic behaviour of reduced glutathione at the dropping mercury electrode

2.4.2.1 Effect of buffer pH

pH is known to influence the oxidation of reduced glutathione in an important extent. Most methods to date use acidic buffer (citrate) : however, the effect of pH on the determination of reduced glutathione was determined by monitoring the peak intensity as well as the shape of the oxidation peaks (Figure 2.8, Appendix 2.1).

2.4.2.1.1 Materials and Method

Solutions of reduced glutathione 50 x 10^{-6} mol.l^{-1} in citrate (pH 3.13 to 4.99), phosphate (pH 7.17-7.95) and sodium chloride/sodium hydroxide/Glycine (pH 8.41-11.22) were prepared.

The determination of glutathione was then carried out by square-wave voltammetry, with a voltage amplitude of 50 mV, a time step of 0.5 s, a measurement time of 0.5 ms, a frequency of 100 Hz. The oxidation peak was obtained during the sweep from 0 to + 0.9 V vs. Ag-AgCl, with a voltage step of 4 mV, and a sweep rate of 8 $ms.s^{-1}$.

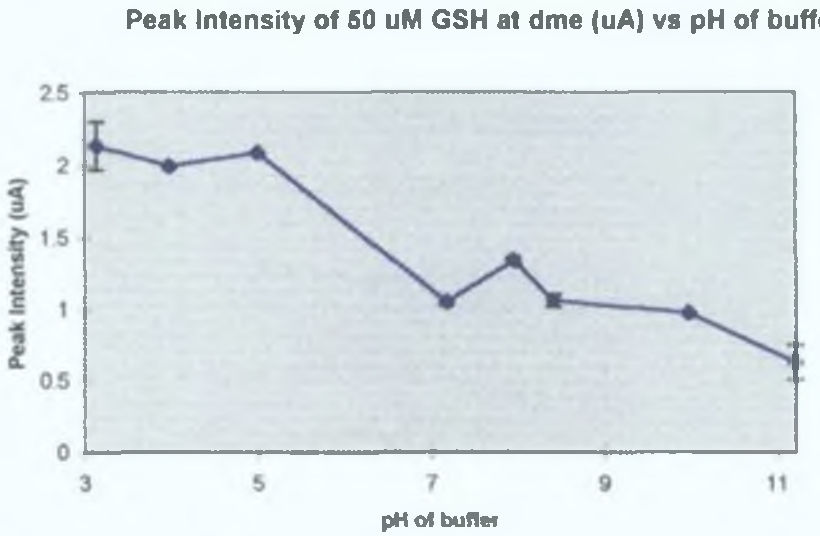
The polarographic instrument was a Metrohm 693 VA-Processor, 694 VA-Stand, with a Ag-AgCl 3 mol l^{-1} reference, and a Glassy Carbon auxiliary electrode.
Square-wave voltammetry was chosen over differential pulse polarography due to its greater sensitivity towards reduced glutathione.

2.4.2.1.2 Results

The Figure 2.8 shows the effect of the pH of the buffer on the peak current for the oxidation of reduced glutathione by square-wave voltammetry at the dropping mercury electrode.

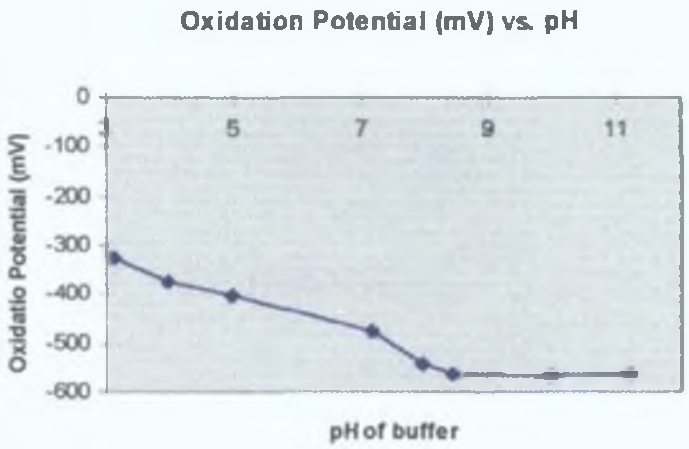
Optimal intensity and peak shape are obtained between pH 3 and 5 (Appendix 2.1). Higher pH lead to poor peak shapes (large width and poor resolution) and are therefore not appropriate for the analysis of reduced glutathione by square wave voltammetry at a dropping mercury electrode.

Figure 2.8 Effect of the pH of buffer on the peak intensity (μA) of 50 μg l^{-1} reduced glutathione in 10^{-2} mol l^{-1} buffer by square-wave voltammetry at the dropping mercury electrode (carried out in triplicate).



It is also interesting to note that the higher the pH, the more cathodic the oxidation peak occurs at (Figure 2.9).

Figure 2.9 Oxidation Potential (mV) vs. electrolyte pH



The shift of the oxidation peak towards more cathodic oxidation potential is observed to be greater from pH 3 to pH 8, where increasing the pH of buffer does not have an important impact on the oxidation peak of reduced glutathione.

2.4.2.2 Effect of the concentration of the buffer

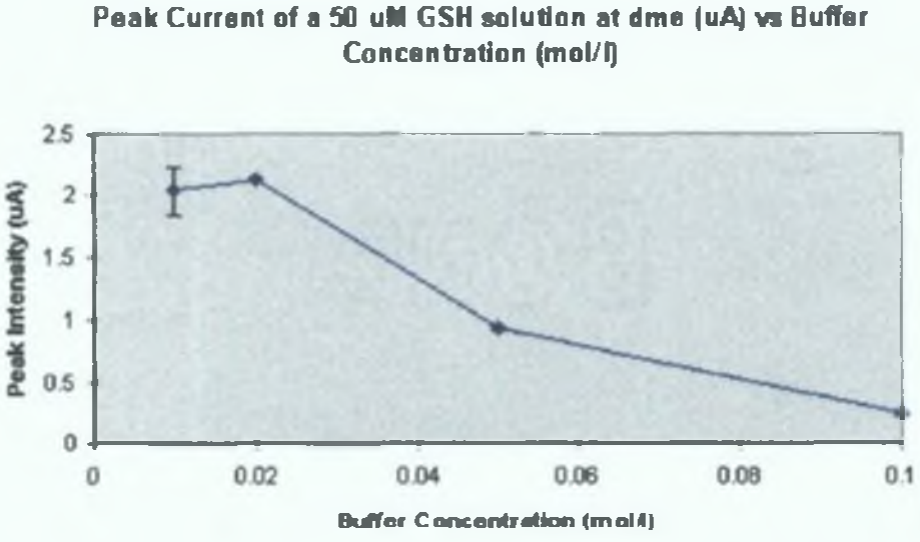
In a similar way, the effect of the concentration of the buffer is an important parameter for electrochemical analysis, and was therefore investigated.

2.4.2.2.1 Materials and Method

Different citrate (pH 4.0) buffer concentrations (from 0.01 to 0.1 mol l^{-1}) were used to prepare solutions containing 5 x 10^{-5} mol l^{-1} reduced glutathione. These solutions were analysed to investigate a possible impact of the buffer concentration on the peak current (Figure 2.10).

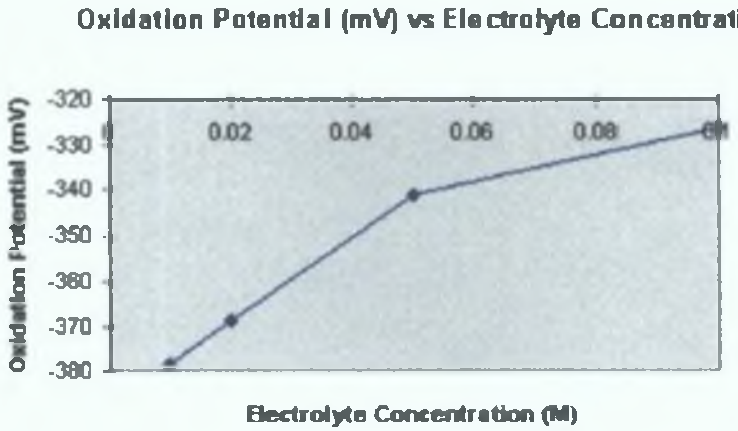
2.4.2.2.2 Results

Figure 2.10 Effect of the concentration of buffer on the peak current (μA) of 50 μg.l^{-1} reduced glutathione by square-wave voltammetry at dropping mercury electrode (carried out in triplicate).



The higher the concentration of the buffer (Figure 2.11), the lower the peak intensity of the oxidation peak of GSH (see Appendix 2.2). The buffer concentration used for future work were therefore set at 10^{-2} mol.l^{-1}.

Figure 2.11 Oxidation Potential (mV) vs. Electrolyte Concentration (mol.l^{-1})



One can also notice that the higher the buffer concentration, the lower negative the voltage occurred.

2.4.2.3 Effect of the pulse time

The effect of the pulse time on the peak intensity of measurement was investigated in order to define the best conditions for the determination of the oxidation of GSH at a dropping mercury electrode, by square wave voltammetry.

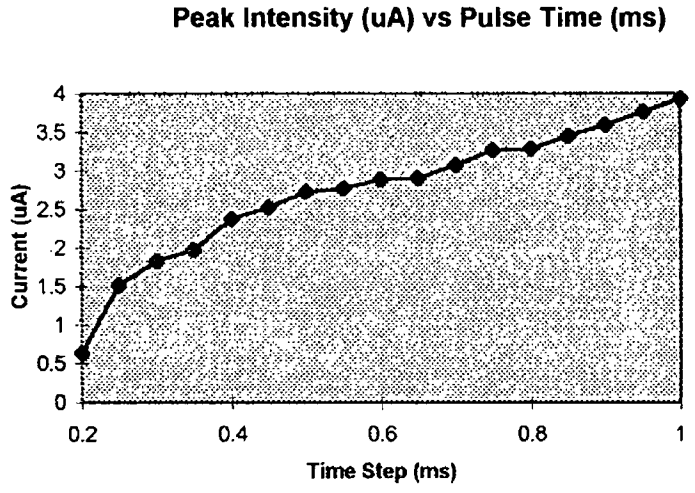
2.4.2.3.1 Materials and Method

The peak currents obtained from the oxidation of a solution containing 50 x 10^{-6} mol.l^{-1} GSH in 10^{-2} mol.l^{-1} citrate buffer pH 4.0 were obtained for pulse time ranging from 0.2 to 1 ms.

2.4.2.3.2 Results

The peak currents obtained from the oxidation of a solution containing 50 x 10^{-6} mol.l^{-1} GSH in 10^{-2} mol.l^{-1} citrate buffer pH 4.0 were plotted versus the pulse time (ms) defined in the method to study the effect of this parameter (Figure 2.12).

Figure 2.12 Effect of the pulse time (ms) on the peak intensity (μA) of 50 μg.l^{-1} reduced glutathione by square-wave voltammetry at a dropping mercury electrode.



The higher the pulse time, the more oxidation occurs, and therefore the higher will the oxidation peak be (see Appendix 2.3). However, the higher the time step, the longer the analysis, and it is therefore important to maximise the pulse time to obtain satisfying sensitivity without increasing the analytical time too much.

The time step had an important effect on the shape of the oxidation peak obtained for GSH. These peaks were poorly shaped at low time step, whereas they were gaussian shaped between 0.4 and 0.8 ms.

2.4.2.4 Determination of the effect of the modulation frequency of the signal pulse on the GSH response

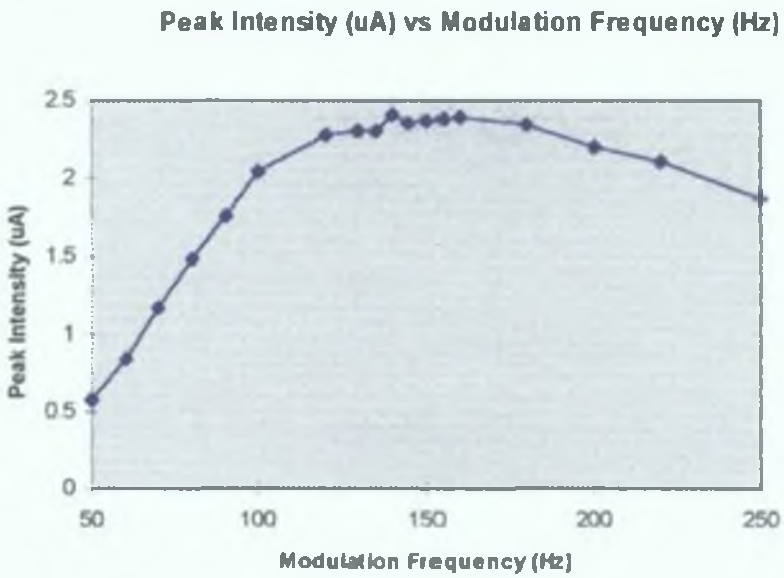
2.4.2.4.1 Materials and Method

The investigation of the effect of the modulation frequency of the signal pulse on the intensity of the peak produced by the oxidation of a solution 5 x 10^{-5} mol.l^{-1} GSH in 2 x 10^{-2} mol.l^{-1} citrate buffer pH 4.0 by square wave voltammetry at a dropping mercury electrode was investigated.

2.4.2.4.2 Results

The peak intensities obtained for the 5 x 10^{-5} mol l^{-1} GSH (Appendix 2.4) were plotted against the modulation frequency to determine the optimum frequency (Figure 2.13) :

Figure 2.13 Effect of the modulation frequency (Hz) of the signal pulse on the peak intensity (μA) of 50 μg l^{-1} reduced glutathione by square-wave voltammetry at a dropping mercury electrode.



An optimum peak intensity was obtained in this case for a modulation frequency range from 130 to 160 Hz. The analytical modulation frequency was set up at 140 Hz for future analysis.

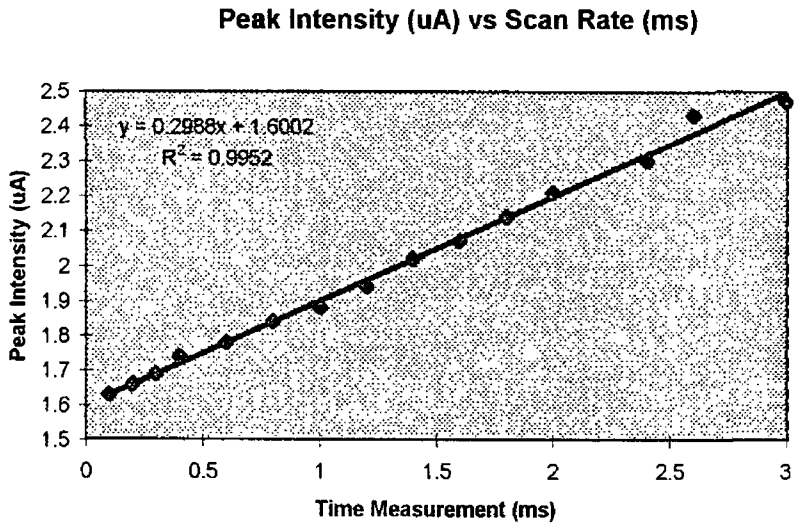
2.4.2.5 Determination of the effect of the scan rate on the GSH response

2.4.2.5.1 Materials and Method

The effect of the scan rate was also investigated for the determination of a solution containing 5 x 10^{-5} mol.l^{-1} GSH in 2 x 10^{-2} mol.l^{-1} citrate buffer pH 4.0 by square wave voltammetry at a dropping mercury electrode (Figure 2.14).

2.4.2.5.2 Results

Figure 2.14 Effect of the scan rate (ms) on the peak intensity (μA) of 50 μg.l^{-1} reduced glutathione by square-wave voltammetry at a dropping mercury electrode.



From the results in Appendix 2.5 and the Figure 2.14, it can be observed that the greater the scan rate, the greater the peak intensity observed for the oxidation of reduced glutathione.

A linear calibration was obtained in this case for scan rate between 0.1 to 3 ms, yielding in a correlation coefficient of 0.9952 with sensitivity of 0.2988 10^{-3} A.s^{-1}. Therefore, the faster the scan rate, the greater the current obtained for the analysis of reduced glutathione by square-wave voltammetry at a dropping mercury electrode.

2.4.2.6 Calibration of a solution of GSH in 2 x 10^{-2} mol.l^{-1} citrate buffer pH 4.0 by square wave voltammetry at a dropping mercury electrode (DME)

2.4.2.6.1 Materials and Method

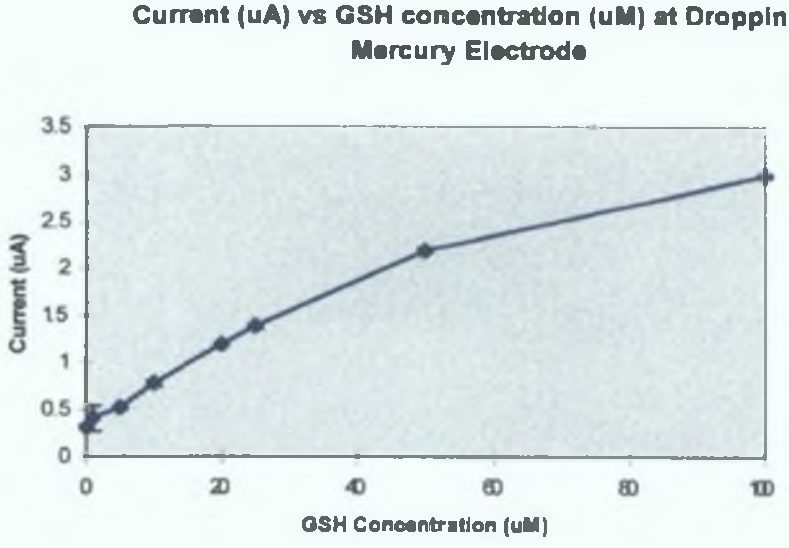
Solutions of GSH in 2 x10^{-2} mol.l^{-1} citrate buffer pH 4.0 were freshly prepared (as GSH oxidises at the atmospheric air) and their calibration was investigated.

The voltammetric parameters during this calibration were chosen from all the previous studies, to attain maximum sensitivity. The voltage amplitude chosen was 50 mV, the modulation frequency of the signal of 140 Hz, the pulse time was 0.5 ms, a Scan Rate of 1 ms and the Voltage Step was 4 mV.

2.4.2.6.2 Results

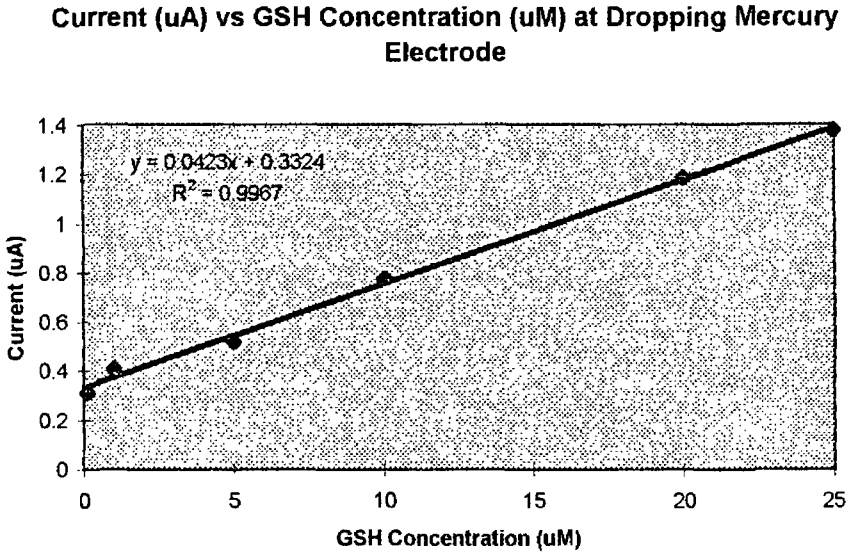
In order to define the dynamic range of the method of determination for GSH by square wave voltammetry (Figure 2.15), the average peak intensities obtained during the previous calibration were plotted against the concentration of GSH in the 10^{-2} mol l^{-1} citrate buffer pH 4.0.

Figure 2.15 Calibration of reduced glutathione (0.1 to 100 x 10^{-6} mol l^{-1}) by square-wave voltammetry at dropping mercury electrode (triplicate).



From the results in Appendix 2.6 and the Figure 2.15, it can be seen that the peak intensities obtained for the oxidation of GSH appear to increase linearly for concentration in GSH ranging from 0.1 to 25 x 10^{-6} mol.l^{-1}, so that a calibration graph could be plotted for the oxidation of GSH at a dropping mercury electrode by square wave voltammetry, according to the previously mentioned parameters (Figure 2.16).

Figure 2.16 Linear calibration of reduced glutathione (0.1 to 25 x 10^{-6} mol.l^{-1}) by square-wave voltammetry at dropping mercury electrode.



A linear calibration for the determination of reduced glutathione (GSH) was therefore obtained by square wave voltammetry at the dropping mercury electrode (Appendix 2.6) between 0.1 and 25 x 10^{-6} mol.l^{-1}. This calibration showed a correlation coefficient r^2 of 0.9964 with a sensitivity of 0.0423 A.mol^{-1}.l.

2.4.2.7 Investigation of the calibration of GSH response by cyclic voltammetry at a hanging mercury electrode by square wave voltammetry

2.4.2.7.1 Materials and Method

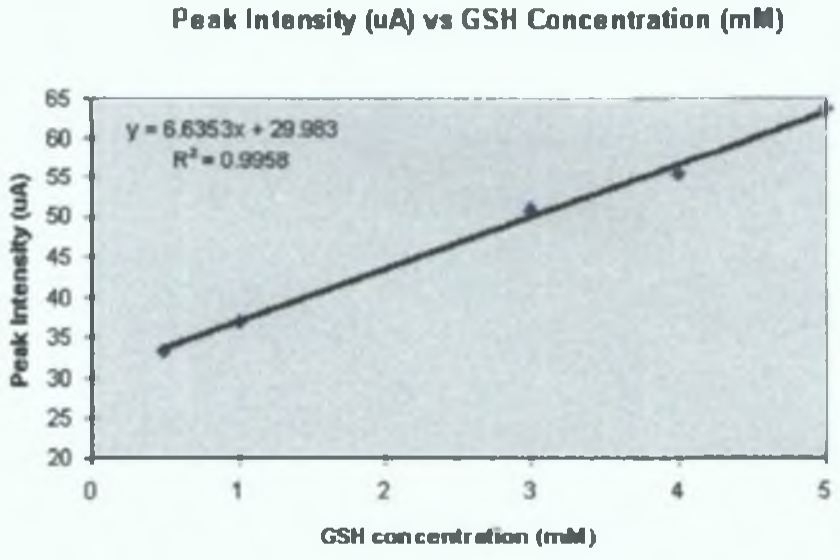
In order to investigate the influence of the concentration in GSH on the oxidation peak obtained by cyclic voltammetry, GSH containing standards were made up in 2 x 10^{-2} mol.l^{-1} citrate buffer and their peak intensities were recorded by square wave voltammetry.

The voltage amplitude chosen was 50 mV, with a voltage step of 0.5 s, a measurement time of 0.5 ms, a voltage step of 4 mV. The modulation frequency of the voltage signal was set at 140 Hz, and an intensity range of 140 µA.

2.4.2.7.2 Results

From the results obtained in Appendix 2.7, the peak intensities obtained for the oxidation of GSH were plotted against the concentrations in GSH, and a linear calibration was defined (Figure 2.17).

Figure 2.17 Linear calibration of reduced glutathione (0.5 to 5 x 10^{-4} mol.l^{-1}) by square-wave cyclic voltammetry at a hanging mercury electrode.



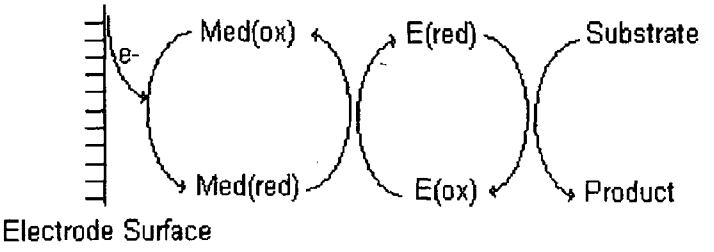
A linear calibration was obtained also in this case within the concentration range from 0.5 and 5 x 10^{-3} mol.l^{-1}. The correlation coefficient r^2 obtained was 0.9958 and the sensitivity was 6.6353 10^{-3} A.mol^{-1}.l.

One can note that the sensitivity of the hanging mercury electrode towards reduced was much lower than obtained for the dropping mercury electrode.

2.4.3 Electron Mediators

Electron mediators are low molecular weight organic compounds that shuttle electrons between the redox centre of the enzyme and the working electrode. Ideally, a mediator for the use in an electrochemical device should react quickly with the enzyme, possess reversible heterogeneous kinetics and have a low overpotential for regeneration.

Figure 2.18 Reaction scheme depicting the role of an electron mediator (E is the enzyme, and Med the mediator)[107].



Ferrocene is used widely as an electron mediator, and its role in the mediation of Glucose Oxidase (GODred) is as follows :

$$GOD_{red} + 2FeCp_2R^+ \rightarrow GOD_{ox} + 2FeCp_2R + 2H^+$$

$$2FeCp2R \leftrightarrow 2\ FeCp2R^+ + 2e^-$$

2.4.4 Modification of glassy, ultra-trace, silver, platinum and gold rotating disk electrodes with ferrocene

The aim of this part of the project was to investigate the effect of modifying the material of the working electrodes (gold, glassy-carbon, ultra-trace, silver and platinum) with a non water-soluble electron mediator, ferrocene.
Ferrocene commonly acts as an electron mediator, and induces the oxidation of reduced glutathione at the different electrode surfaces.

The tip of the glassy-carbon, silver, gold and platinum electrodes were first polished with 2 μm alumina, and then carefully cleaned with ultra-pure water, to attain smooth surfaces.
In the case of the ultra-trace electrode, the tip of the electrode was renewed everyday using the ceramic scrapper at disposal by metrohm to get a fresh new outer surface.

After the polishing or scrapping of the electrodes, which aimed at creating a renewed electrode surface, these were modified by applying 10 μls volumes of 10^{-1} mol.l^{-1} ferrocene solution in toluene onto their surfaces. The toluene was then allowed to evaporate at room temperature, the ferrocene staying on the electrodes surface as a thin smear.

2.4.5 Modification of glassy, ultra-trace and gold rotating disk electrodes with mercury-film

The glassy-carbon and gold rotating disc electrodes were pre-treated in the same way than in 2.4.3.

Mercury-modified electrodes were obtained using a mercury Hg^{2+} solution of 10^{-1} mol.l^{-1} potassium nitrate and 5 x 10^{-3} mol.l^{-1} mercuric nitrate in ultra-pure water. The deposition step of the mercury layer at the electrode surface was carried out applying a potential of - 1.0 V vs. Ag-AgCl 3 mol.l^{-1} for 2 minutes.

The electrodes were cleaned at + 1.5 V vs. Ag-AgCl for 5 minutes after use to remove the mercury from the surfaces. In the case of the ultra-trace electrode, the scrapping of the outer layer was carried out to ensure total renewal of the surface.

2.5 Investigation of the behaviour of reduced and oxidised glutathione by cyclic voltammetry

2.5.1 Materials and Method

The cyclic behaviour of reduced glutathione at various types of rotating-disc electrodes was determined by Square-wave Cyclic Mode. The voltage amplitude was 50 mV, with a Sweep Rate of 25 $mV.s^{-1}$, a modulation frequency of 140 Hz, and an intensity range of 140 μA. The pulse time was 2 s, with a voltage step of 5 mV, a scan rate of 0.2 ms.
2 x 10^{-2} $mol.l^{-1}$ citrate buffer pH 3.0, 6 x 10^{-2} $mol.l^{-1}$ reduced glutathione (GSH) and 6 x 10^{-2} $mol.l^{-1}$ oxidised glutathione (GSSG) in the previous buffer were prepared.

The polarographic instrument was a Metrohm 693 VA-Processor, 694 VA-Stand, with a Ag-AgCl 3 mol^{-1} reference electrode. Several rotating disc electrodes were used, such as glassy-carbon, ultra-trace epoxy-graphite, and silver.
The possibility of using a mercury-film deposited on the surface of the ultra-trace and the gold electrodes at a potential of -1.0 V vs. Ag-AgCl for 300 s was also investigated.

2.5.2 Results at the glassy-carbon rotating disc-electrode

From the cyclic voltammograms obtained, it was observed that no oxidation or reduction occurred at a noticeable level at the surface of the glassy-carbon electrode.

The glassy-carbon rotating disc electrode was therefore modified by the addition of ferrocene, an electron mediator, at the surface of the electrode. However, the cyclic voltammograms did not change in any way.

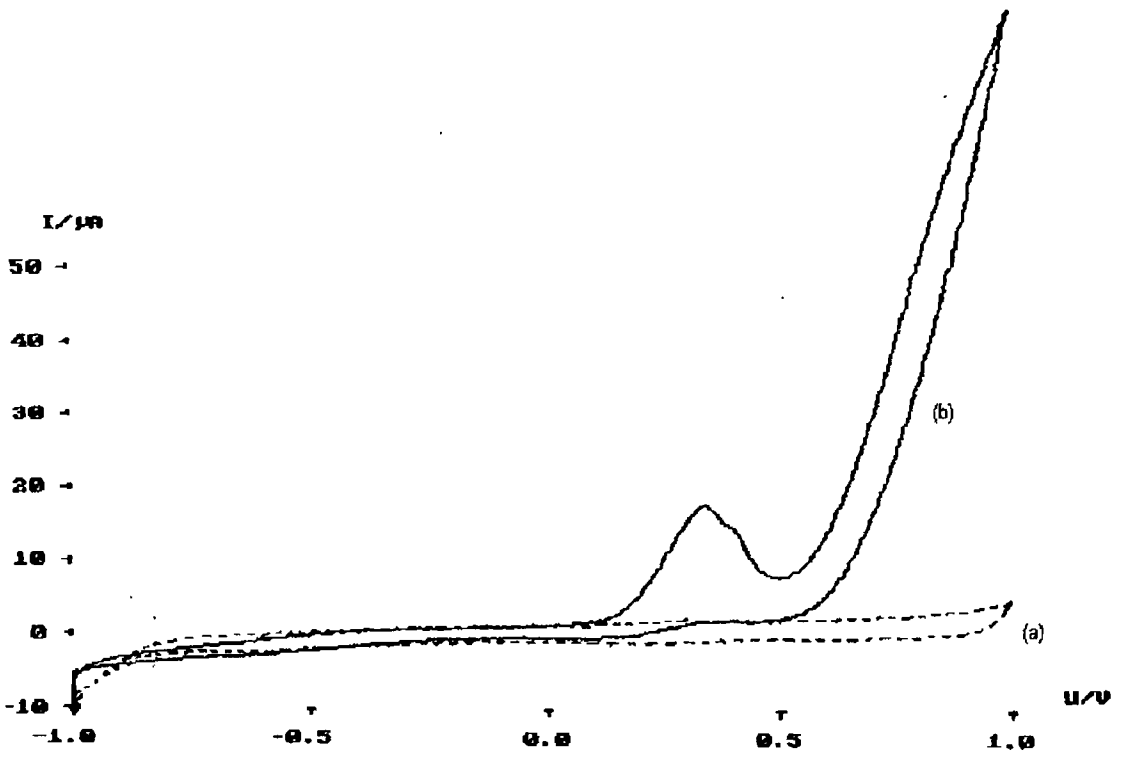
However, the cyclic voltammograms were not affected in any way, and no oxidation or reduction peak were obtained.

It was therefore concluded that modified and unmodified glassy-carbon electrodes were not suitable electrode material for the determination of glutathione by square wave cyclic voltammetry.

2.5.3 Results obtained at the ultra-trace epoxy-graphite rotating disc electrode

Cyclic voltammograms of 2 x 10^{-2} mol.l^{-1} citrate buffer pH 3, 6 x 10^{-2} mol.l^{-1} oxidised glutathione (GSSG) and finally 6 x 10^{-2} mol.l^{-1} reduced glutathione (GSH) were obtained between - 1.0 to + 1.0 V vs. Ag-AgCl at the ultra-trace RDE (Figure 2.18).

Figure 2.18 Cyclic voltammograms of a) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 b) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 + 6 x 10^{-2} mol.l^{-1} reduced glutathione at the ultra-trace epoxy-graphite electrode.



No oxidation nor reduction peak were observed for the cyclic voltammetry of the citrate buffer on its own. The addition of GSSG to the citrate buffer increased the magnitude of the intensities at - 1.0 and + 1.0 V vs. Ag-AgCl. However, no reduction peak was obtained as expected in the negative sweep.
A broad oxidation peak of intensity 13 μA is observed in the positive sweep of the GSH solution at + 0.33 V vs. Ag-AgCl.

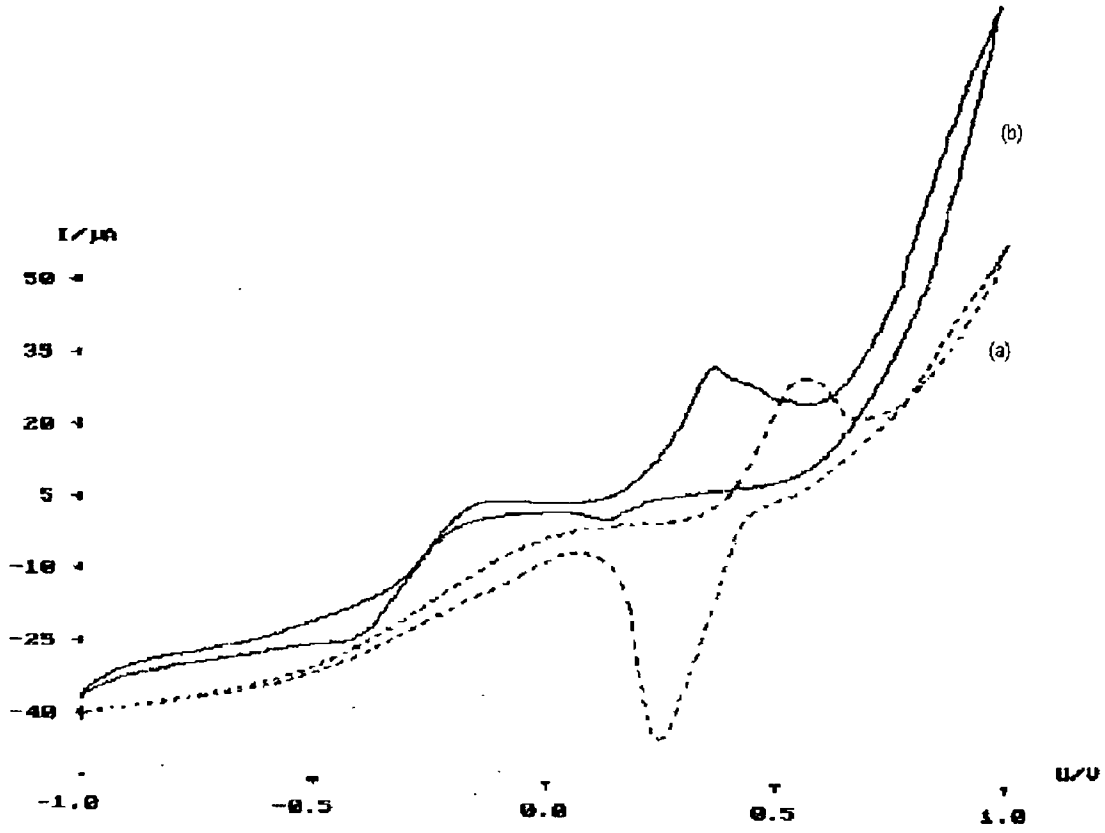
2.5.4 Results obtained at the mercury-modified ultra-trace electrode

The cyclic voltammogram obtained for 2 x 10^{-2} mol.l^{-1} citrate buffer pH 3.0 (Figure 2.19) shows an oxidation peak at + 0.565 V vs. Ag-AgCl of 16.25 μA during the positive sweep. On the reverse scan, a large reduction peak is obtained at + 0.24 V vs. Ag-AgCl (42.5 μA).

The cyclic voltammogram obtained for the 6 x 10^{-2} mol.l^{-1} GSSG solution in citrate buffer showed a small oxidation peak at + 0.3 V (6.25 μA) and a small reduction at + 0.075 V vs. Ag-AgCl (1.25 μA).

In the same way, an oxidation and a reduction peak were observed at + 0.375 V vs. Ag-AgCl (17.5 μA) on the positive sweep and at + 0.14 V (2.5 μA) for the cyclic voltammetry of a solution of 6 x 10^{-2} mol.l^{-1} GSH in 2 x 10^{-2} mol.l^{-1} citrate buffer.

Figure 2.19 Cyclic voltammograms of a) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 b) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 + 6 x 10^{-2} mol.l^{-1} reduced glutathione at the mercury-modified ultra-trace electrode



This type of electrode cannot effectively be used for the determination of GSH as GSSG also shows an oxidation peak on the positive sweep (+ 0.3 V and + 0.375 V vs. Ag-AgCl respectively).

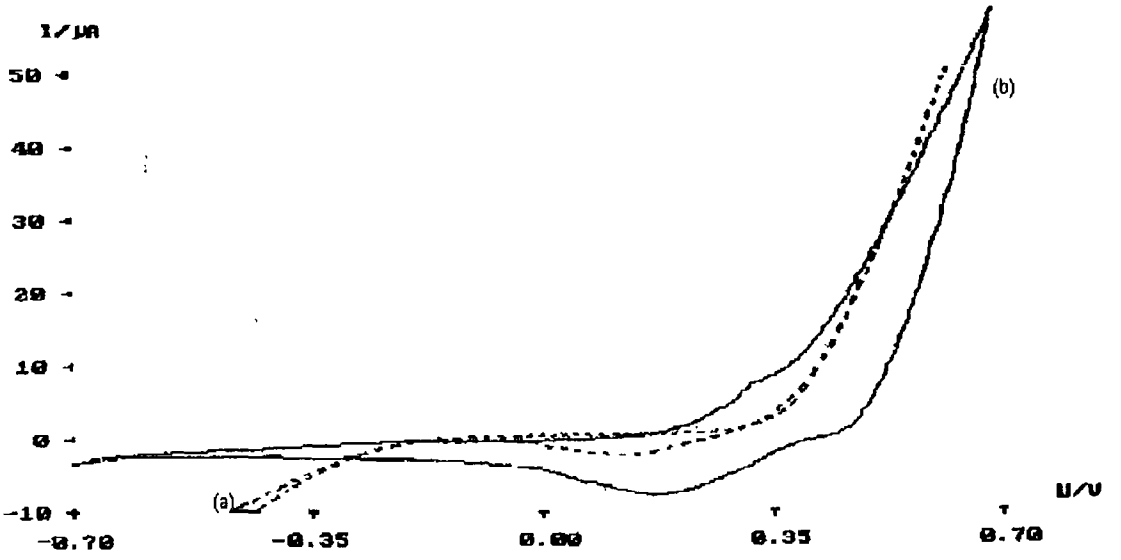
2.5.5 Results obtained at the silver rotating disc electrode

The cyclic voltammetric behaviour of the 2 x 10^{-2} mol.l^{-1} citrate buffer pH 3.0 was studied between - 0.7 V and + 0.7 V vs. Ag-AgCl by cyclic voltammetry. No noticeable oxidation nor reduction peak were obtained in this case.

A reduction peak was observed for the negative sweep of 6 x 10^{-2} mol.l^{-1} GSSG at a potential of + 0.1 V vs. Ag-AgCl (7.80 µA), which corresponds to its reduction to GSH (figure 2.20).

In the case of 6 x 10^{-2} mol.l^{-1} GSH, an oxidation peak is obtained at + 0.32 V vs. Ag-AgCl while a reduction peak occurs at + 0.185 V. The peak intensities are difficult to determine in this case, however, the current obtained appeared to be subsequently larger than for GSSG or the buffer (Figure 2.20).

Figure 2.20 Cyclic voltammograms of a) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 b) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 + 6 x 10^{-2} mol.l^{-1} reduced glutathione at a silver electrode.



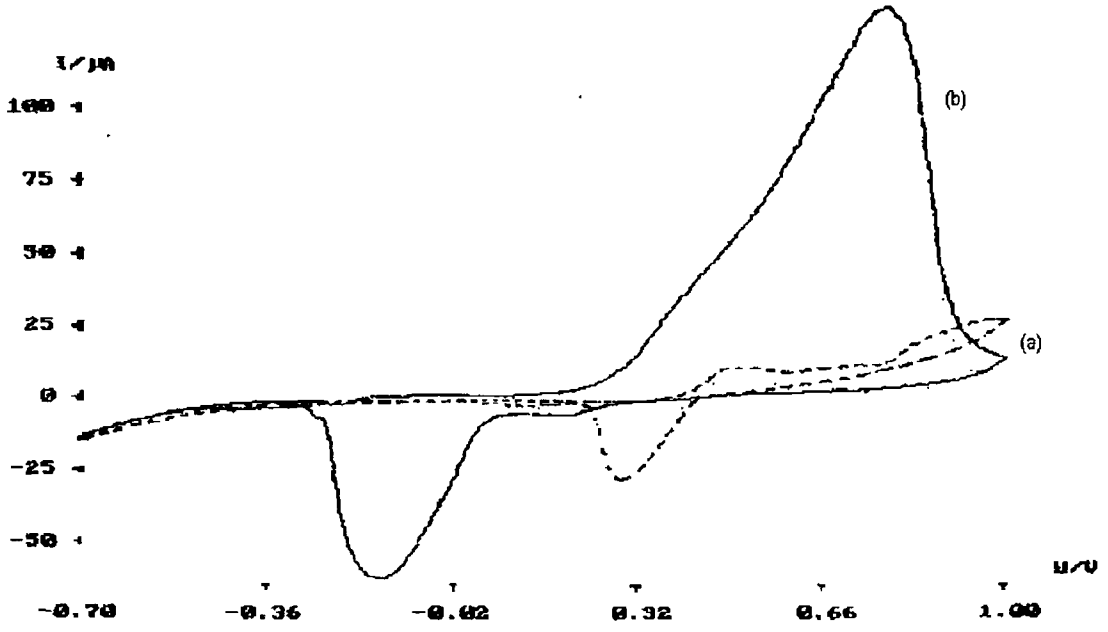
From such results, one can conclude that reduced glutathione can be successfully analysed at the rotating silver electrode.

2.5.6 Results obtained at the mercury-gold amalgam rotating disc electrode

The cyclic voltammogram obtained for the 2 x 10^{-2} mol.l^{-1} citrate buffer pH 3.0 between - 0.7 V and + 1.0 V vs. Ag-AgCl showed a small oxidation peak (6.20 μA) on the positive sweep at + 0.495 V and a bigger reduction peak (24.65 μA) on the negative sweep at + 0.295 V vs. Ag-AgCl (Figure 2.21).

In the case of 6 x 10^{-2} mol.l^{-1} GSSG, the oxidation peak observed on the positive sweep of the buffer disappears whereas the reduction peak observed on the negative sweep switches to + 0.67 V vs. Ag-AgCl (30.80 μA).
The oxidation peak for 6 x 10^{-2} mol.l^{-1} GSH was obtained at + 0.78 V vs. Ag-AgCl (123 μA) in the positive sweep, whereas a reduction peak occurred at - 0.122 V (58.6 μA) the negative sweep (Figure 2.21).

Figure 2.21 Cyclic voltammograms of a) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 b) 10^{-2} mol.l^{-1} citrate buffer pH 3.0 + 6 x 10^{-2} mol.l^{-1} reduced glutathione at a mercury-gold amalgam electrode.



This electrode can be successfully investigated for the determination of GSH by square wave voltammetry as the peak intensity obtained for this later is larger than for the buffer.

2.5.7 Cyclic voltammetric analysis of reduced glutathione :

Cyclic voltammetric investigations of the behaviour of reduced glutathione, oxidised glutathione and the citrate buffer were conducted at a range of different rotating disc electrodes.

Paradoxically, glassy carbon electrode could not be used successfully, as no oxidation or reduction could be observed for either GSH or GSSG. Ferrocene was therefore used as an electron mediator, however unsuccessfully.

The ultra-trace epoxy-graphite electrode rotating disc electrode showed no reduction nor oxidation for either the buffer nor GSSG. However, GSH showed a clear and important oxidation peak at + 0.33 V vs. Ag-AgCl. Therefore, this electrode material could be successfully used for the oxidation of GSH by square wave voltammetry.
When modified with a mercury film, the epoxy-graphite material shows a higher background current and therefore is not satisfactory for this type of analysis.

The mercury-gold modified rotating disc electrode showed high oxidation and reduction peaks for GSH and therefore could be used for the determination of GSH by square-wave cyclic voltammetry.

The silver material was the last to be investigated and showed an important difference between the GSH and GSSG, but however could not be of use for the determination of GSH by square-wave cyclic voltammetry.

2.6 Development of the immobilised glutathione s-transferase reactor for on-line conjugation of organochlorine compounds with GSH

2.6.1 Immobilisation of glutathione s-transferase (GsT) on aminopropyl porous glass beads :

Glutathione s-transferase, porous amino propyl glass beads (170 and 700 Å), glutaraldehyde, Tris(HCl), bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene, ethacrynic acid, 2,4-dichloronitrobenzene (CDNB) purchased from Sigma-Aldrich.

Glutathione s-transferase was immobilised on aminopropyl porous glass beads (170 and 700 Å). This immobilisation step was essential in the development of an immobilised enzyme reactor. Such procedure allows the continuous use of the enzyme over a certain length of time.

The first step of this immobilisation process (Figure 2.7) was carried out by adding 10 ml of 5 % glutaraldehyde to 1 g of 1:1 aminopropyl porous glass beads (170:700 Å, mixture to prevent high back pressure in the column).

The mixture was then placed under vacuum for 45 minutes and rotated for a further 2 hours. The beads were then washed with approximately 200 ml of ultra-pure water, to remove any trace of glutaraldehyde, which would affect the enzyme activity. 2 mg of glutathione s-transferase in 1 x 10^{-3} $mol.l^{-1}$ Tris.HCl pH 7.0, were added to the beads which were rotated at room temperature for a further 15 hours. Finally, 100 mg of bovine serum albumin were added to the beads, which were rotated for another 2 hours.
This last step was carried out to react any un-reacted groups at the beads surface.
The beads were then thoroughly washed with approximately 200 ml of ultra-pure water (conductivity < 10 μS) to remove any unattached enzyme and excess bovine serum albumin.

2.6.2 Investigation of the activity of the immobilised glutathione s-transferase towards 1-chloro-2,4-dinitrobenzene, 2,4-dichloro-nitrobenzene and ethacrynic acid - Preparation of an enzyme reactor

2.6.2.1 Materials and Method

Glutathione s-transferase, porous amino propyl glass beads (170 and 700 Å), glutaraldehyde, Tris(HCl), bovine serum albumin, 1-chloro-2,4-dinitrobenzene, ethacrynic acid, 2,4-dichloronitrobenzene (Sigma-Aldrich).

The beads containing the immobilised GsT as prepared in 2.6.1 were packed into an Omnifit liquid chromatography column (10 mm x 100 mm), whose ends were carefully blocked with glass wool, to ensure the beads were tightly packed.
A blank reactor was prepared in the same way, except that no GsT were added in 2.6.1.

The conjugations of 1-chloro-2,4-dinitrobenzene, 2,4-dichloronitrobenzene, and ethacrynic acid, with reduced glutathione through the enzyme reactor, were qualitatively analysed by ultraviolet/visible spectrophotometry.

In order to test the enzyme reactor prepared, a set of solutions containing 1×10^{-3} mole.l^{-1} (a) 1-chloro-2,4-dinitrobenzene, (b) 2,4-dichloronitrobenzene, (c) ethacrynic acid, also containing 2.5×10^{-3} mol.l^{-1} GSH were prepared in 1×10^{-2} mol.l^{-1} phosphate buffer pH 7.0.

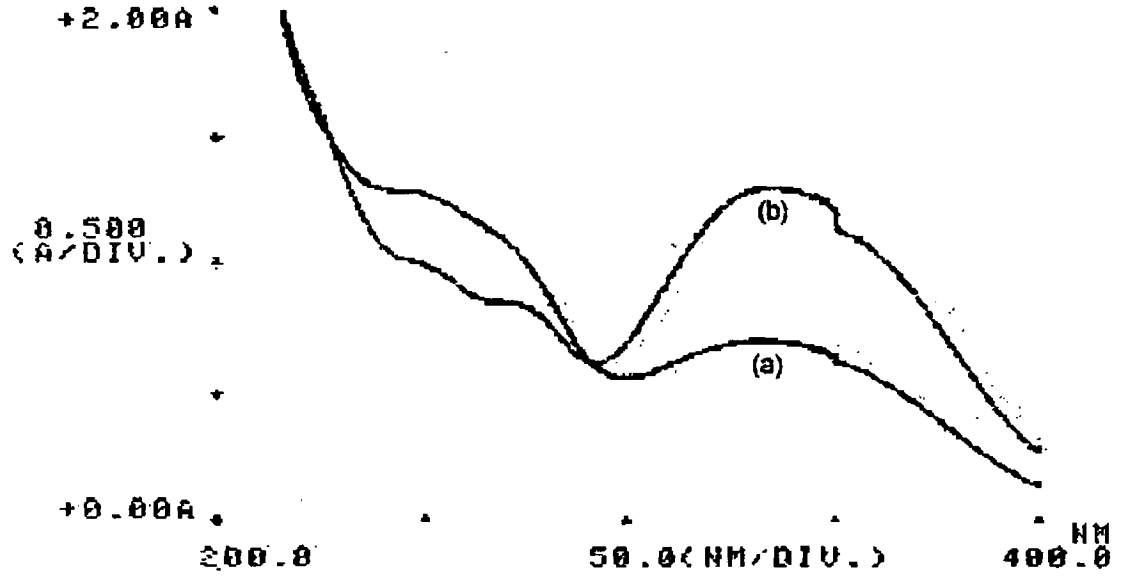
Each solution was eluted at a flow rate of 2 ml.min^{-1} through the enzyme reactor in the following manner : 10 ml of 1×10^{-2} mol.l^{-1} phosphate buffer pH 7.0 was eluted to condition the enzyme reactor (the last 5 ml of which were collected), then by 20 ml of the sample solutions, followed by another 10 ml of the phosphate buffer (the first 5 ml of which were also collected).

In a similar way, each solution was eluted through the blank reactor. The two sets of solutions obtained (each sample being approximately 30 ml) were studied qualitatively by UV and by HPLC.

2.6.2.2 Spectrophotometry

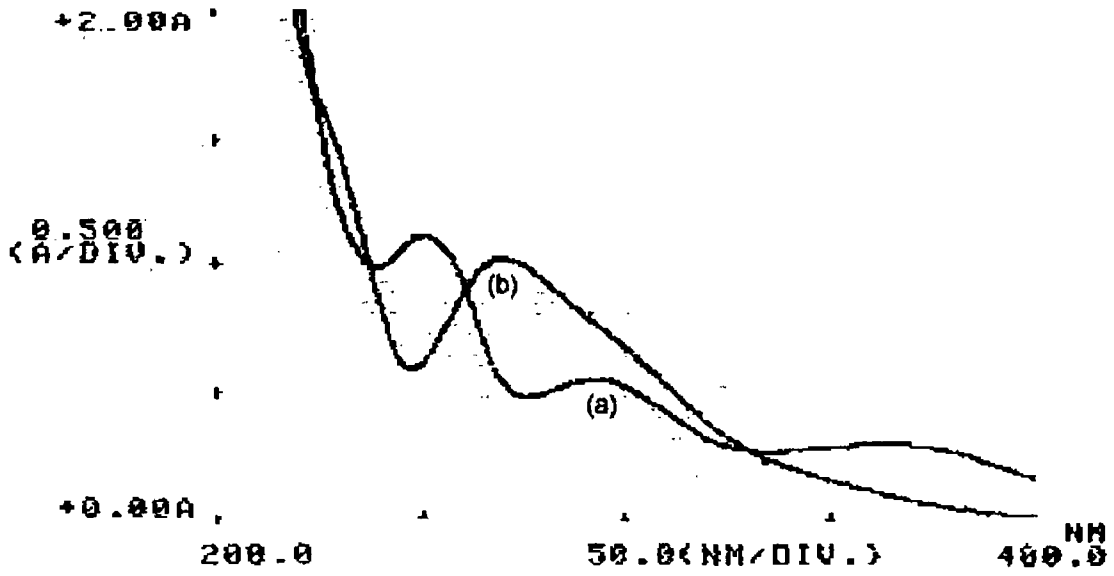
UV spectra were obtained for each samples between 200 and 400 nm on a UV/Visible UV-160A spectrophotometer (Shimadzu).

Figure 2.22 Ultraviolet/visible spectra of 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene in 2.5 x 10^{-3} mol.l^{-1} reduced glutathione in 0.01 mol.l^{-1} phosphate buffer pH 7.0, a) before and b) after elution through glutathione s-transferase.



When comparing the spectra obtained for the elution products through the blank and GsT immobilised reactor, one can notice the appearance of a greater absorbance at 330 nm in the latest (Figure 2.22). This shows the evidence of a greater conjugation between CDNB and GSH when eluted and put in contact with the immobilised glutathione s-transferase than when put in contact with blank beads. However, it is noticeable that some conjugation is obtained : the enzyme in fact speeds up the reaction which would occur at a smaller speed when not catalysed.

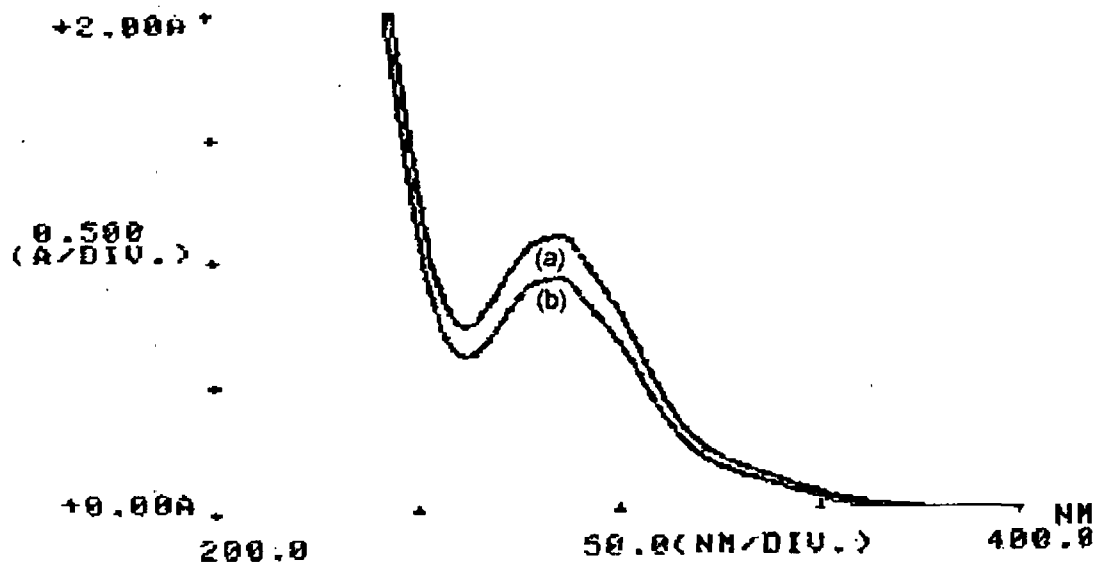
Figure 2.23 Ultraviolet/visible spectra of 10^{-3} mol.l^{-1} 2,4-dichloronitrobenzene in 2.5×10^{-3} mol.l^{-1} reduced glutathione in 0.01 mol.l^{-1} phosphate buffer pH 7.0; a) before and b) after elution through glutathione s-transferase.



In the case of 2,4-dichloronitrobenzene (DNB), a change in spectra was also observed upon elution through the enzyme immobilised reactor when compared to the blank reactor (Figure 2.23). These results suggest the evidence that the conjugation between DNB and GSH (observed at 270 nm) occurred when in contact with the enzyme immobilised in the enzyme reactor. In this case no conjugation product was observed in the elution products from the blank reactor, mainly due to the fact that the speed of reaction between DNB and GSH is lower than between CDNB and GSH.

However, in the case of ethacrynic acid, no significant change in the spectra obtained for the elution of GSH/ethacrynic acid through the blank and the enzyme immobilised reactor could be observed. This suggests that the enzyme reactor cannot catalyse the conjugation between ethacrynic acid and GSH during their elution.

Figure 2.24 Ultraviolet/visible spectra of 10^{-3} mol.l^{-1} ethacrynic acid in 2.5 x 10^{-3} mol.l^{-1} reduced glutathione in 1 x 10^{-2} mol.l^{-1} phosphate buffer pH 7.0, a) before and b) after elution through glutathione s-transferase.



2.6.3 Quantitative analysis of the conjugation product of CDNB with GSH by UV :

The conjugation of CDNB with GSH was carried out through the elution of sample solutions of different concentration in CDNB in the enzyme reactor. Quantitative analysis was important in this case to determine if the enzyme immobilised in the reactor reacted to a range of different concentrations of CDNB.

2.6.3.1 Materials and Method

A set of solutions containing 1-chloro-2,4-dinitrobenzene concentrations of 0, 0.1, 0.2, 0.4, 0.6 and 0.8 x 10^{-3} mol.l^{-1} was prepared in 10^{-2} mol.l^{-1} Tris.HCl pH 7.0 / 10^{-3} mol.l^{-1} reduced glutathione. A test solution only containing 0.8 x 10^{-3} mol.l^{-1} was prepared in 1 x 10^{-2} mol.l^{-1} Tris.HCl, pH 7.0.

These solutions were eluted at a flow rate of 4 ml.min^{-1}, through the described glutathione s-transferase enzyme reactor prepared in 2.6.1. The column was previously conditioned with 10 ml of 1 x 10^{-2} mol.l^{-1} Tris.HCl, pH 7.0 buffer (the last 5 ml of which were collected), followed by 20 ml of the sample (from the set of solutions prepared above), followed by another 10 ml of buffer (the first 5 ml of which were also collected). The set of eluted samples was then tested by UV at 330 nm.

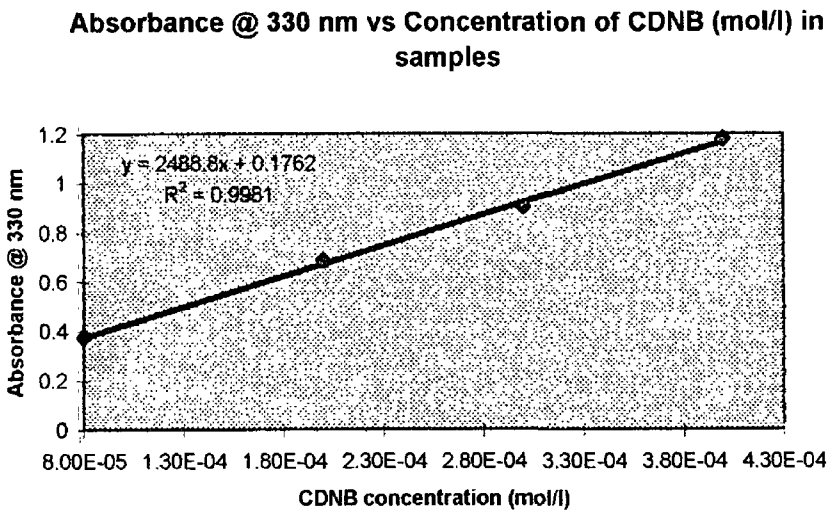
2.6.3.2 Results

The spectra obtained for this calibration can be observed in Figure 2.25.

Figure 2.25 Ultraviolet/visible spectra of the conjugation product of 1-chloro-2,4-dinitrobenzene (8 x 10^{-5} mol.l^{-1} to 4 x 10^{-4} mol.l^{-1}) with 10^{-3} mol.l^{-1} reduced glutathione, after elution through the glutathione s-transferase reactor.

From the results observed in Figure 2.25 and the Appendix 2.8, a linear calibration was be plotted (Figure 2.26), and sensitivity of 2488.8 absorbance.mol^{-1}.l., intercept and correlation determined (0.9981).

Figure 2.26 Linear calibration of the Absorbance @ 330 nm of the conjugation product of 1-chloro-2,4-dinitrobenzene (8×10^{-5} mol.l^{-1} to 4×10^{-4} mol.l^{-1}) with 10^{-3} mol^{-1} reduced glutathione, after elution through the glutathione s-transferase reactor.



2.6.4 Qualitative determination of the conjugation of CDNB product with GSH by HPLC

2.6.4.1 Materials and Method

The 1-chloro-2,4-dinitrobenzene, ethacrynic and 2,4-dichloronitrobenzene solutions prepared for the qualitative determination of the conjugates obtained with GSH upon elution were analysed by HPLC.

LC-10 AS, coupled with a SPD-10A ultraviolet/visible detector (Shimadzu).
Column : Allsphere ODS-1 54 (250 mm x 4.6 mm I.D.).
Mobile phase : 49.5:49.5:1 % Methanol : Water : Acetic Acid.
Flow rate : 1 $ml.min^{-1}$.

These solutions were first eluted at a flow rate of 4 $ml.min^{-1}$ through the glutathione s-transferase containing enzyme reactor as described previously. The column was conditioned with 10 ml of 1 x 10^{-2} $mol.l^{-1}$ Tris.HCl, pH 7.0 buffer (the last 5 ml were collected), followed by 20 ml of sample (from the set of solutions mentioned above), followed by another 10 ml of buffer (the first 5 ml were also collected).

A set of eluted samples (approximately 30 ml each) was therefore collected and tested by HPLC (@ 254 and 330 nm).

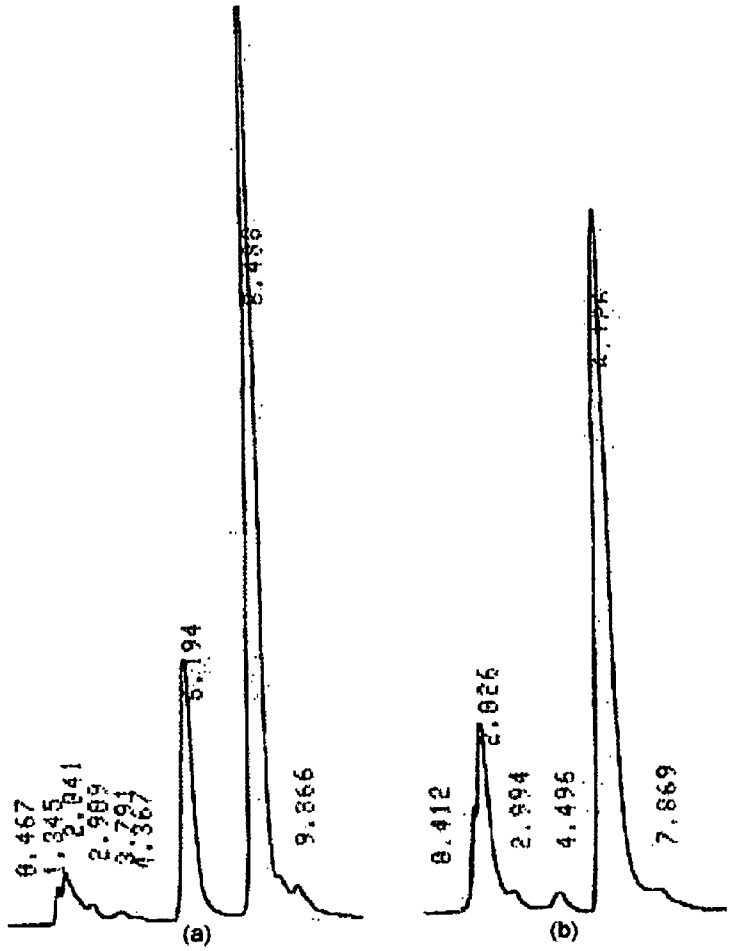
2.6.4.2 Results

The analysis of the eluate obtained for the solution of CDNB after elution through the enzyme reactor as described above were compared with the eluate obtained after elution through a blank reactor.

The chromatograms obtained in Figure 2.27 show that the conjugation of CDNB with GSH (6.12 minutes) appeared greater after elution through the enzyme containing reactor than through the blank reactor. However, one can noticed that

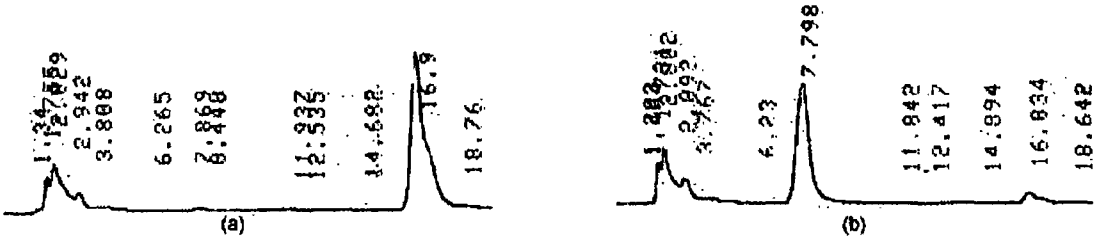
the analysis of the elution product through the blank reactor showed evidence of conjugation product.

Figure 2.27 High performance liquid chromatograph of 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene with 2.5 x 10^{-3} mol.l^{-1} reduced glutathione in 10^{-2} mol.l^{-1} phosphate buffer pH 7.0 a) before and b) after elution through glutathione s-transferase reactor. Mobile phase : 49.5:49.5:1 % (MeOH:H_2O:AcCOOH).



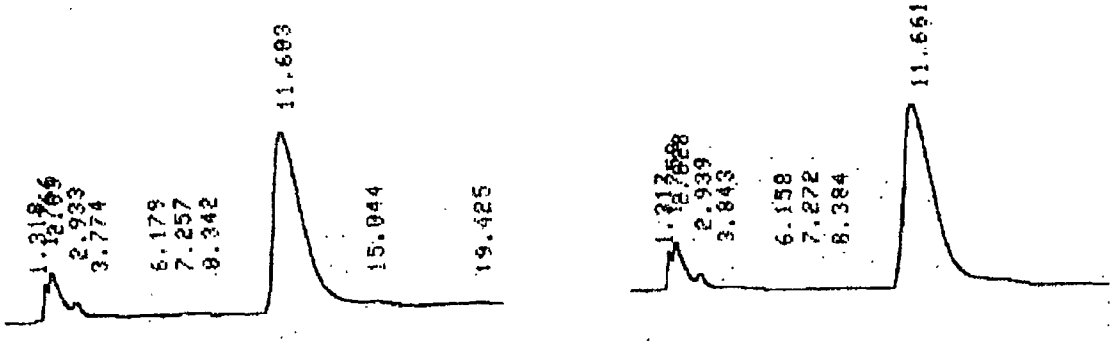
In a similar way, the elution products obtained for DNB through the blank and enzyme containing reactors were compared in Figure 2.27. These chromatograms show the evidence of the conjugation being obtained between GSH and DNB (7.8 minutes) through the enzyme immobilised reactor, rather than in the blank reactor.

Figure 2.27 High performance liquid chromatograph of 10^{-3} mol.l^{-1} 2,4-dichloronitrobenzene with 2.5 x 10^{-3} mol.l^{-1} reduced glutathione in 10^{-2} mol.l^{-1} phosphate buffer pH 7.0 a) before and b) after elution through glutathione s-transferase reactor. Mobile phase : 49.5:49.5:1 % (MeOH:H_2O:AcCOOH).



The same analysis was carried out for ethacrynic acid, however no change could be observed after elution through the blank or the enzyme reactor, proving that the results previously obtained by UV/VIS.

Figure 2.28 High performance liquid chromatograph of 10^{-3} mol.l^{-1} ethacrynic acid with 2.5 x 10^{-3} mol.l^{-1} reduced glutathione in 10^{-2} mol.l^{-1} phosphate buffer pH 7.0 a) before and b) after elution through glutathione s-transferase reactor. Mobile phase : 49.5:49.5:1 % (MeOH:H_2O:AcCOOH).



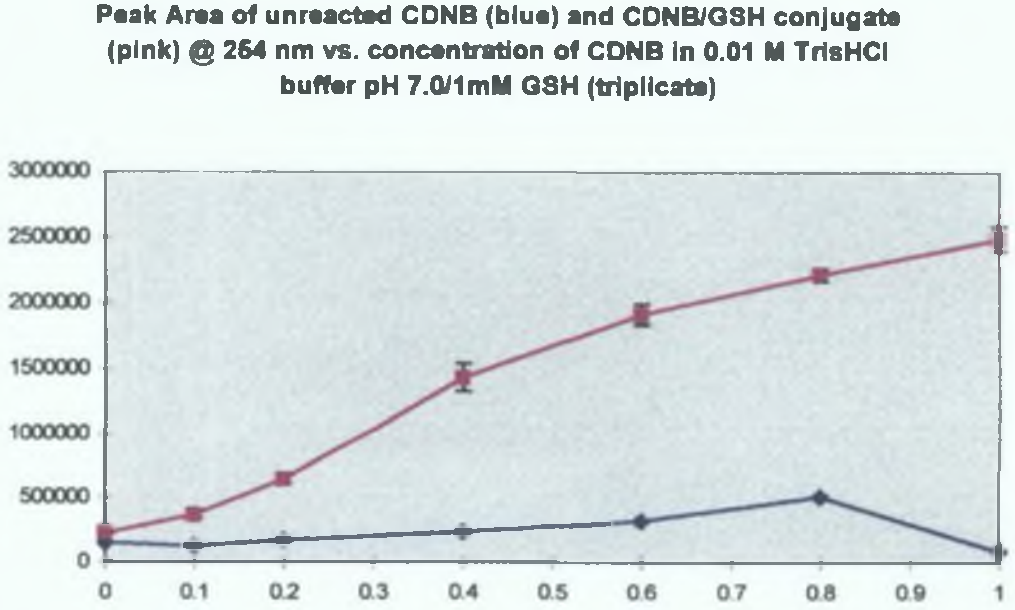
2.6.4.3 Quantitative analysis of CDNB conjugate with GSH by HPLC

2.6.4.3.1 Materials and Method

A set of solutions containing concentrations of 0, 0.1, 0.2, 0.4, 0.6 and 0.8 x 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene was prepared in a 1 x 10^{-2} mol.l^{-1} Tris.HCl pH 7.0 / 1 x 10^{-3} mol.l^{-1} GSH buffer solution. A test solution only containing 0.8 x 10^{-3} mol.l^{-1} CDNB was prepared in 1 x 10^{-2} mol.l^{-1} Tris.HCl, pH 7.0 buffer. The results obtained can be observed in Appendix 2.9.

2.6.4.3.2 Results

Figure 2.29 Calibration curve obtained for a range of 1-chloro-2,4-dinitrobenzene (from 0 to 0.8 x 10^{-3} mol.l^{-1}) in constant concentration of reduced glutathione (1 x 10^{-3} mol.l^{-1}) after elution through glutathione s-transferase reactor, analysed by high performance liquid chromatography, at 254 nm.
Mobile phase : 49.5:49.5:1 % MeOH:H_2O:AcCOOH.



2.6.5 Electrochemical investigation of the conjugation of CDNB with GSH

2.6.5.1 Materials and Method

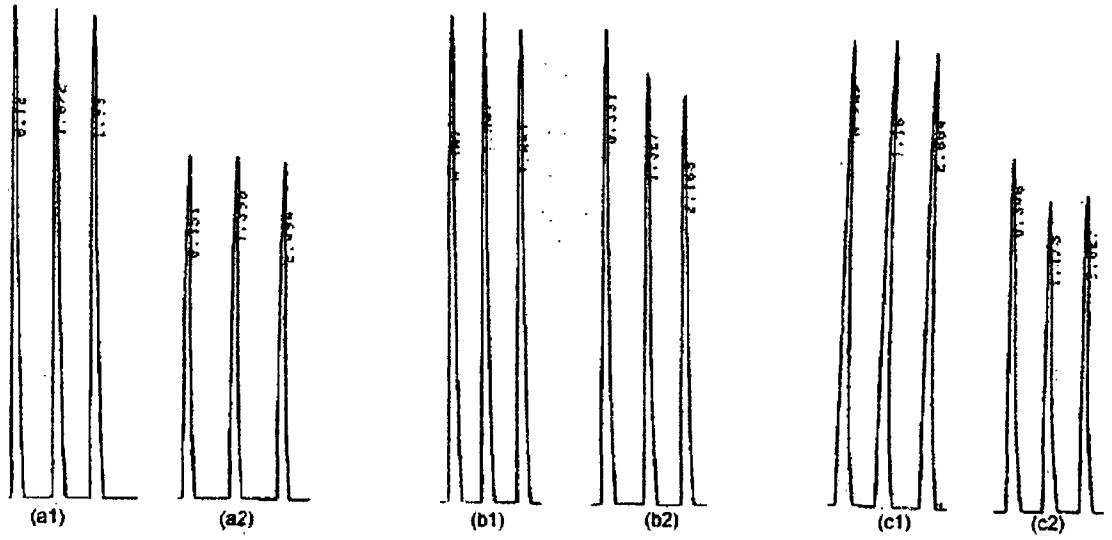
A 1.5 x 10^{-3} mol.l^{-1} solution of GSH in phosphate buffer (1 x 10^{-2} mol.l^{-1}, pH 7.0) was prepared and injected into a flow-through electrochemical cell (BAS CC-5, Cross-Flow, BAS Technicol) with phosphate buffer as electrolyte. Three solid electrode materials (gold, glassy carbon and silver) were tested for the electrochemical determination of GSH at + 0.9 V vs. Ag-AgCl, to experimentally optimise the analysis.

Another solution containing 1.5 x 10^{-3} mol.l^{-1} solution of GSH in phosphate buffer (1 x 10^{-2} mol.l^{-1}, pH 7.0) and 1 x 10^{-3} mol.l^{-1} was gravity eluted through the enzyme reactor developed in 2.6.1. The eluted solution was then also electrochemically analysed at gold, glassy carbon and silver electrodes (+ 0.9 V vs. Ag-AgCl).

A set of GSH standards in the concentration range 2 x 10^{-3} to 5 x 10^{-5} mol.l^{-1} were also prepared in phosphate buffer, and analysed at + 0.9 V vs. Ag-AgCl at the glassy carbon electrode.

2.6.5.2 Electrochemical oxidations of glutathione at the glassy-carbon, gold and silver electrode

Figure 2.30 Amperometric oxidation of a 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene and 1.5 x 10^{-3} mol.l^{-1} reduced glutathione in 10^{-2} mol.l^{-1} phosphate buffer pH 7.0, at + 0.9 V vs. Ag-AgCl, before elution through the aminopropyl porous glass beads glutathione s-transferase reactor respectively (triplicate)
a1) glassy-carbon electrode, a2) enzyme reactor / glassy-carbon,
b1) gold electrode, b2) enzyme reactor / gold,
c1) silver electrode, c2) enzyme reactor / silver electrode.



One can observed from the results in Appendix 2.10 and the Figure 2.30 that the differences between each electrode material upon the electrochemical determination of GSH and the CDNB/GSH conjugate obtained after elution through the enzyme reactor.

From the results obtained before the elution through the reactor, it can be concluded that glassy-carbon and gold would be suitable materials for the analysis of GSH in the flow-cell , with RSD of 1.02 and 1.00 % respectively. The RSD obtained for the same solution at the silver material showed higher at 2.72 % and therefore should not be used over the other two types.

After elution of the CDNB and GSH containing solution through the enzyme reactor, an electrochemical change was observed. The intensity of the peaks obtained decreased suggesting a change in the chemistry of the solution.

This could be due to the formation of a non electrochemical product, which would yield in the decrease of the total peak area. This non-electrochemical product could be the conjugation product analysed by UV and HPLC in 2.6.3 and 2.6.4.

The deviation between each material varied greatly this time between glassy-carbon and silver and gold, RSD being respectively 0.38, 9.76 and 9.85 %.

As a result, glass-carbon was chosen as the material of use for further analysis of GSH and its conjugate with CDNB.

2.6.5.3 Calibration of glutathione at the glassy-carbon electrode

The calibration of the set of solution containing from 5 x 10^{-5} to 2 x 10^{-2} mol.l^{-1} GSH was therefore obtained in Figure 2.31. A calibration curve was then plotted from the results in Appendix 2.11, yielding in a slope of 1.5 x 10^{9} uV.mol^{-1}.l, the intercept 741.1, and a correlation factor of 0.9986 (Figure 2.32).

Figure 2.31 Calibration of a set of solution containing from 2 x 10^{-3} to 5 x 10^{-5} mol.l^{-1} GSH in a 1 x 10^{-2} mol.l^{-1} phosphate buffer, pH 7.0, amperometrically analysed at + 0.9 V vs. Ag-AgCl, at a glassy-carbon.

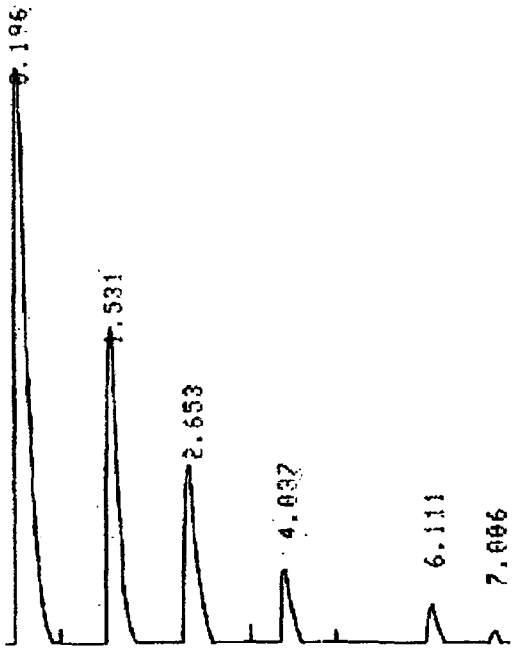
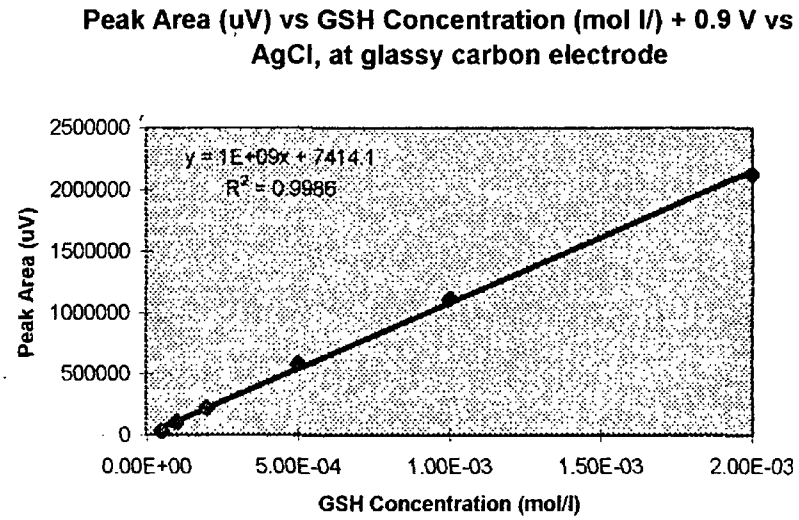


Figure 2.32 Calibration was obtained at the glassy carbon electrode for a set of solution containing from 2×10^{-3} to 5×10^{-5} mol.l^{-1} GSH in a 1×10^{-2} mol.l^{-1} phosphate buffer, pH 7.0.

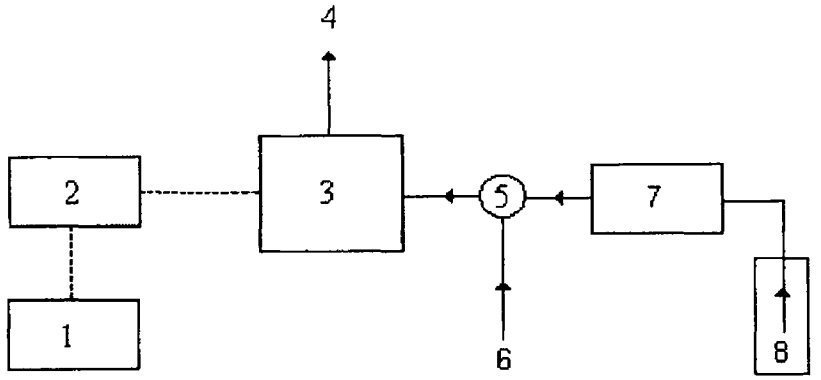


2.6.5.4 Flow-through system

2.6.5.4.1 Materials and Method

The bioreactor was placed into a flow-through system as schematised in Figure 2.33.

Figure 2.33 Instrumental schematic



1	Integrator	5	Injection valve
2	Potentiostat	6	Sample inlet
3	Flow-cell / Electrochemical Detector	7	Peristaltic pump
4	Waste	8	Electrolyte

A set of solutions consisting of 0, 0.1, 0.2, 0.4, 0.6 and 0.8 x 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene were prepared in a 1 x 10^{-2} mol.l^{-1} Tris.HCl pH 7.0 containing 1 x 10^{-3} mol.l^{-1} GSH.

The solutions were injected through the reactor column at a flow rate of 4 ml.min^{-1}, as described in 2.6.2, and off-line electrochemical detection followed the elution through the enzyme reactor at the glassy carbon at + 0.9 V vs. Ag-AgCl. The results obtained were plotted on the Figure 2.34.

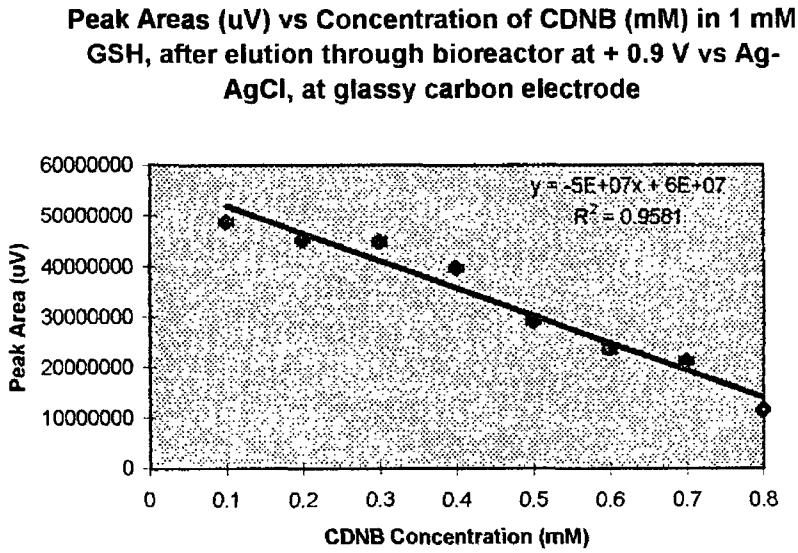
2.6.5.4.2 Results

The results in Appendix 2.12 and the calibration plot in Figure 2.34 show the evidence that the greater the concentration in CDNB in the solution before elution, the lower the oxidation in the eluted solutions.

The electrochemical compound present in the solutions before elution changes to a non-electrochemical compound after elution through the bioreactor. This suggests that the conjugation between CDNB and GSH forms a non-electrochemical compound.

One can also observed a trend in the decrease, showing a linear relationship between the increase of the concentration in CDNB and the increase of conjugation catalysed by the enzyme reactor.

Figure 2.34 Amperometric calibration of solutions of 1-chloro-2,4-dinitrobenzene from 0.1 to 0.8 x 10^{-3} mol.l^{-1} in 10^{-3} mol.l^{-1} reduced glutathione in 10^{-3} mol.l^{-1} Tris.HCl, after elution through the glutathione s-transferase reactor. Determined at a glassy carbon electrode, at + 0.9 V vs. Ag-AgCl.



2.6.5.5 On-line determination of the conjugate

The next step in the development of the analysis of CDNB on-line by conjugation with GSH, and electrochemical determination at the silver electrode, was to investigate if adding GSH to the buffer would be possible. The aim of this step was to determine if a constant signal could be obtained for GSH, which would be altered upon conjugation with CDNB, which would be directly injected prior to the enzyme reactor. A Rheodyne Omnifit injection valve with a loop of 1.2×10^{-4} dm^3 was used to inject the sets of solutions.

Solutions of 1-chloro-2,4-dinitrobenzene from 0.1 to 0.8 x 10^{-3} $mol.l^{-1}$ in 10^{-3} $mol.l^{-1}$ reduced glutathione in 10^{-3} $mol.l^{-1}$ Tris.HCl, were prepared for this purpose. They were then injected directly through the rheodyne just prior to the enzyme reactor placed between the injector valve and the electrochemical detector (Figure 2.33).

This analysis was not feasible due to an increase in the background noise from the GSH present in the buffer, resulting in electrode fouling, which rendered the flow-cell unusable. The only change observed in the signal was a constant drift towards zero response due to the fouling or caking of the electrode tip of the electrochemical detector.

2.7 Discussion

As described above, the capacity of the immobilised enzyme to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene, 2,4-dichloronitrobenzene and ethacrynic acid with reduced glutathione was investigated by ultraviolet-visible spectrophotometry, HPLC and electrochemical detection.

Ultra-violet spectrophotometric analysis

From the spectra obtained, the conjugation of CDNB with GSH catalysed by the immobilised glutathione-s-transferase was observed in the eluates @ 330 nm. A much smaller conjugation was observed in the case of 2,4-dichloronitrobenzene @ 345 nm.

However, nothing could be observed in the eluate of ethacrynic acid. This could be due to the highest molar extinction coefficient value $\Delta\varepsilon$ (9.6 $mM^{-1}.cm^{-1}$) of CDNB over the other two compounds (8.5 and 5.0 x 10^{-3} $mol.l^{-1}.cm^{-1}$ for 2,4-dichloronitrobenzene and ethacrynic respectively), or that the conjugation did not occur to the same extent.

A linear calibration was obtained for the conjugation of the samples of 1-chloro-2,4-dinitrobenzene ranging from 8 x 10^{-5} $mol.l^{-1}$ to 4 x 10^{-4} $mol.l^{-1}$ in phosphate buffer pH 7.0, and in presence of 10^{-3} $mol.l^{-1}$ reduced glutathione, after their elution through the enzyme reactor.

HPLC

For the separation of the above samples by HPLC, a peak was separated for a solution of 1 x 10^{-3} $mol.l^{-1}$ CDNB and 2.5 x 10^{-3} $mol.l^{-1}$ GSH in 1 x 10^{-2} $mol.l^{-1}$ phosphate buffer. After 1 day at room temperature, this peak was observed to be even greater. A peak is clearly obtained for CDNB at 8.488 min, and a second peak is observed at 6.194 min. However, this peak can't be accounted for GSH, which does not absorb at 330 nm. This peak could however be due to the conjugation between CDNB and GSH. The conjugation of CDNB with GSH can occur without catalysis, however, glutathione s-transferase speeds up the reaction.

The analysis of the same standard solution after elution through the enzyme reactor showed no peak at 8.488 min, but an increase of the size of the peak at 6.13 min. All the CDNB present in the solution had disappeared, and therefore, the peak at 6.19 min can be attributed to the conjugate between CDNB and GSH. Therefore, much greater conjugation was observed when CDNB and GSH were put in contact with the enzyme, during their elution through the enzyme immobilised reactor.

In a similar manner, 2,4-dichloronitrobenzene (DNB) eluted at 16.9 min; however no other peak was observed. After elution through the enzyme reactor, a peak was obtained at 7.80 min, which would account for the conjugate of DNB with GSH, as the peak observed at 16.9 min had disappeared.

No conjugation was however observed for ethacrynic acid by HPLC, both chromatograms displaying peaks at 11.6 min.

UV/VIS and HPLC analyses showed the ability of the glutathione s-transferase reactor to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene and 2,4-dichloronitrobenzene to reduced glutathione under the specified conditions ($2ml.min^{-1}$, 1×10^{-2} $mol.l^{-1}$ phosphate buffer, pH 7.0). However, no evidence was observed of any conjugation between ethacrynic acid and reduced glutathione. This was surprising as ethacrynic acid has been extensively used as substrate for the determination of the activity of glutathione s-transferase.

Quantitative analysis of the CDNB/GSH conjugate by HPLC

The following step in the project was the quantitative investigation by HPLC of the conjugation between 1-chloro-2,4-dinitrobenzene with reduced glutathione after elution through the enzyme reactor. A fixed concentration of GSH was therefore used (1×10^{-3} $mol.l^{-1}$) and variable amounts of CDNB were eluted through the enzyme reactor.

In this case, the conjugation reaction was carried out at a higher flow rate (4 $ml.min^{-1}$) in order to speed up the time of analysis.

The conjugation reaction was not complete as some CDNB was still observed in the eluted samples. Another column was also used in this case, leading in different retention times. CDNB was eluted at 5 min, and the conjugate at 12.9 min, which suggests the lower polarity of the conjugate than CDNB. The sets of solutions were analysed both at 254 nm and 330 nm, the latest showing higher absorbance and therefore peak area for CDNB and its conjugate with GSH.

It was observed that, with a constant concentration of GSH (1×10^{-3} $mol.l^{-1}$), the increase of CDNB (from 0 to 0.8×10^{-3} $mol.l^{-1}$) yields in an increase in the conjugate peak in the eluates.

Electrochemical behaviour of GSH

The electrochemical behaviour of GSH at glassy carbon, gold and silver electrode was investigated. The gold and glassy carbon electrodes displayed similar sensitivity towards GSH, while the sensitivity of the silver electrode was lower. Glassy carbon and gold electrodes also gave more reproducible peak heights with relative deviation of 1.02 % and 1.00 % respectively, compared to 2.72 % for silver.

Electrochemical behaviour of the conjugate

Passing the solution of GSH alone, and with CDNB through the enzyme reactor, prior to injection into the electrochemical flow-cell, resulted in a 33.91 % and 32.96 % decrease in the signal obtained at the glassy carbon and gold electrodes respectively. However, the decrease observed at the silver electrode was only 6.21 %. High sensitivity towards GSH at the working electrode is sought to detect the smallest amounts of conjugation between GSH and CDNB. The highest sensitivity of glassy carbon and gold materials towards GSH, as well as their reproducibility (1.02 and 1.00 % respectively), compared to those attained with the silver material, induced the choice of glassy carbon electrode over silver. Glassy carbon was chosen over gold for its ease of cleaning. The determination of

GSH at the glassy-carbon was then investigated, and a linear calibration obtained between 5 x 10^{-5} to 2 x 10^{-3} mol.l^{-1}.

Quantification of the conjugate

The determination of the conjugation of CDNB (0 to 0.8 x 10^{-3} mol l^{-1}) with GSH (1 x 10^{-3} mol l^{-1}) was monitored after the enzyme reactor by spectrophotometry (i.e. the determination of the conjugate at 330 nm) and by electrochemistry at a glassy carbon at + 0.9 V vs. Ag-AgCl.

However, CDNB nor its complex with GSH possess any electrochemical properties at + 0.9 V vs. Ag-AgCl, so that changes in GSH concentrations must be monitored. By setting a constant concentration of GSH (1 x 10^{-3} mol l^{-1}) in the set of solutions, and varying the concentration of CDNB (from 0 to 0.8 x 10^{-3} mol l^{-1}), CDNB could be determined as a decrease in the signal given off from GSH.

The samples obtained from the elution of the standards through the enzyme reactor were monitored off-line through the electrochemical cell, at a glassy carbon electrode. The higher the CDNB concentration in an excess GSH (10^{-3} mol l^{-1}), the lower the peak area of the oxidation peak obtained at the glassy carbon electrode. These results were plotted and a linear calibration in the concentration range from 0 and 0.8 x 10^{-3} mol l^{-1} was obtained, with a correlation coefficient r^2 of 0.9581, and a sensitivity of -5 x 10^4 mol^{-1} l.

The linear relationship between the decrease of the peak area obtained with an increase of the CDNB concentration could be accounted for the increase in the complexation catalysed by the immobilised beads. These results suggest the possibility of using immobilised glutathione s-transferase sensors for the determination of CDNB by electrochemistry.

Direct on-line determination of the conjugation product between CDNB and GSH, as catalysed by immobilised GsT present in the reactor, was investigated. However, no significant change in the background signal could be observed. This

could be due to the band broadening of the compounds through the column during the elution.

The same problem was encountered when injecting CDNB on its own, with GSH present in the mobile phase. This was due to the fouling of the electrode occurring rapidly after elution, which was due to the formation of layer of decomposition compounds arising from the oxidation of GSH.

Another project should therefore focus on the development of disposable screen-printed carbon-paste or glassy-carbon electrode, modified by a fixed concentration of glutathione s-transferase and reduced glutathione.

CHAPTER 3.0

CONCLUSION

The undertaken project aimed at the development of sensing systems for environmental monitoring. This was split between the development of a sensing system for the determination of chromium (+VI) and the development of a sensing system for 1-chloro-2,4-dinitrobenzene. Both parts of the projects were introduced through extensive literature reviews covering the essential research and theoretical research carried out up to now.

The electrochemical investigation of the conjugation product between 1,5-diphenylcarbazide and chromium (+VI) was carried out at an epoxy-graphite, chemically-modified carbon-paste and over-oxidised polypyrrole gold electrode.
Cyclic voltammetric study of the conjugate was obtained at the epoxy-graphite electrode, and quantitative analysis by cathodic adsorptive voltammetry was obtained for solutions as low as 1 $ug.l^{-1}$ in chromium (+VI). The method investigated was based on an application study carried out by the supplier of the polarograph, and qualitative and quantitative results were obtained.
The electrochemical determination of chromium (III) was also investigated at the dropping mercury, between 1 to 100 $mg.l^{-1}$.

Of all the types of electrode materials investigated, ultra-trace graphite material proved to the best, because it allowed fast and sensitive determination of hexavalent chromium (down to 1 $ug.l^{-1}$).
For trivalent chromium, adsorptive differential pulse voltammetry (AdSV) at the dropping mercury electrode was found to be the better option. However, this analysis suffered from lower detection limits than the electrochemical method developed for hexavalent chromium.
A very important part of the research was that both methods of analysis allow for speciation of trivalent and hexavalent chromium. This should be used in order to differentiate the hexavalent form, which represents the most threat to health and environment, from the trivalent form, which doesn't.

Future work on this part of the project should focus on the development of an 1,5-diphenylcarbazide modified screen-printed disposable electrode. The

feasibility of using this sensor for the determination of hexavalent chromium should be investigated in depth.

The second part of the project focused on the investigation of the use of glutathione s-transferase (GsT) for the development of an enzyme reactor, capable of determining organochlorine compounds. This enzyme is commonly used for the catalysis of substrates such as 1-chloro-2,4-dinitrobenzene, ethacrynic acid, and 2,4-dichloronitrobenzene, with reduced glutathione (GSH). The objective was to immobilise this enzyme and pack it into a reactor to develop an enzyme reactor. The capacity of this reactor was thoroughly investigated and its capacity to catalyse the conjugation of the 3 substrates with GSH determined.

This was obtained by Ultra-violet and visible spectrophotometry, as well as high-performance liquid chromatography, and electrochemistry, for the three substrates. The reactor was shown to be capable of catalysing the conjugation of 1-chloro-2,4-dinitrobenzene and 2,4-dichloronitrobenzene with GSH. However, it was not capable of catalysing the reaction between ethacrynic acid with GSH. This was probably due to its lower molar extinction coefficient when compared to the other two substrates, hence inducing a longer conjugation.
Quantitation by UV/Vis and HPLC were successfully obtained for the conjugation between 1-chloro-2,4-dinitrobenzene and GSH obtained upon their elution through the GsT immobilised reactor.

The electrochemical behaviour of reduced glutathione was investigated at different chemically-modified and un-modified substrates such as gold, silver, glassy-carbon and carbon-paste. Cyclic voltammetry was carried out to determine the electrode material which would optimal electroanalytical parameters for the determination of GSH.

Of all the electrode materials investigated for the determination of reduced glutathione (glassy-carbon, silver and gold), the results obtained for the glassy-carbon material proved to better than for the others, and was therefore preferred.

In a similar way, the electrochemical of a GSH solution was monitored before and after conjugation with GsT, obtained upon elution through the enzyme containing reactor. Of the three different electrode materials examined for the determination of the product of the conjugation between reduced glutathione and 1-chloro-2,4-dinitrobenzene, glassy-carbon showed better sensitivity and repeatability.

The quantitative conjugation between reduced glutathione and 1-chloro-2,4-dinitrobenzene was electrochemically investigated at the glassy-carbon electrode, and a decrease of the signal upon conjugation was obtained. This is due to the lack of electrochemical nature of the conjugation product, as catalysed by the immobilised glutathione s-transferase. This proved that the immobilisation process as described in the project, can be successfully carried out for the development of an enzyme sensing system, capable of the determination of 1-chloro-2,4-dinitrobenzene.

From the qualitative results obtained, one can suggests that the developed enzyme reactor should be capable of catalysing the conjugation between reduced glutathione and 2,4-dichlorobenzene, but not for ethacrynic acid.
To determine the feasibility of using the developed sensing system for the determination of other organochlorine compounds one would need to investigate thoroughly the electrochemical properties of DNB and its conjugate with GSH, which was not carried out during the project. Ethacrynic acid did not show any conjugation properties with reduced glutathione upon elution through the enzyme immobilised sensing reactor.
A wider electrochemical investigation would need to be carried out to determine if changing characteristics such as the pH and concentration of the buffer, could allow such a conjugation to take place.

Future work on a similar project should also focus on the development of disposable GsT and GSH immobilised carbon-paste sensors, for the determination of 1-chloro-2,4-dinitrobenzene and may be 2,4-dichloronitrobenzene

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APPENDICES

Appendix 1.1

Absorbance at 254 nm vs Concentration of Chromium(VI) in diphenylcarbazide.

Cr(VI) concentration (ug/l)	Absorbance @ 254 nm
0.01	0.001
0.1	0.002
1	0.002
4	0.002
10	0.004
100	0.034
1000	0.73
10000	1.958

Appendix 1.2

Determination of Cr^{3+} (mg l^{-1}) in 1 x 10^{-3} mol l^{-1} EDTA, 0.5 mol l^{-1} $NaNO_3$ and 5 x 10^{-2} mol l^{-1} CH_3COONa, by differential pulse at a dropping mercury electrode.

Cr3+ concentration (mg l-1)	Intensity Peak (mA)	P. Peak (nW)
100	3.09	314
75	1.7	209
20	0.83	81.7
10	0.37	29.2
1	0.029	0.88

Appendix 2.1

Effect of pH on the square-wave voltammetric determination of reduced glutathione (50×10^{-6} mol l^{-1}) in 10^{-2} mol l^{-1} buffer at dropping mercury electrode.

pH	U. Peak (mV)	I. Peak (mA)	P. Peak (nW)	U. Width (mV)
3.13	-326	2.32	215.2	62
	-327	2.01	208.0	65
	-326	2.07	344.1	58
3.98	-374	1.97	192.6	67
	-370	2.01	235.7	66
	-374	1.99	243.9	78
4.99	-404	2.08	210.6	76
	-400	2.09	214.9	65
	-404	2.09	212.1	73
7.17	-478	1.05	109.9	66
	-477	1.03	109.1	67
	-476	1.07	114.9	66
7.95	-545	1.34	148.7	92
	-542	1.31	148.7	87
	-543	1.36	154.2	86
8.41	-567	1.06	122.5	109
	-562	1.02	118.9	99
	-566	1.10	126.0	77
9.96	-570	0.97	117.8	113
	-565	0.98	113.7	96
	-570	0.97	117.4	114
11.2	-564	0.57	56.7	91
	-561	0.55	55.7	85
	-565	0.77	83.3	105

Appendix 2.2

Effect of the citrate buffer concentration (x 10^{-3} mol l-1) on the square-wave voltammetric determination of reduced glutathione (50 x 10^{-6} mol l^{-1}) at dropping mercury electrode.

Buffer Concentration (mol l-1)	U. Peak (mV)	I. Peak (mA)	P. Peak (nW)	U. Width (mV)
0.1	-323	0.22	12.2	41
	-327	0.24	11.89	37
	-330	0.24	12.61	37
0.05	-340	0.95	59.61	43
	-342	0.93	56.35	42
	-342	0.92	58.29	45
0.02	-367	2.13	196.9	64
	-370	2.13	192.3	58
	-369	2.14	198.9	58
0.01	-374	2.01	204.1	60
	-391	1.85	187.0	71
	-371	2.24	203.0	54

Appendix 2.3

Effect of the pulse time (ms) on the square-wave voltammetric determination of reduced glutathione (50×10^{-6} mol l^{-1}) at a dropping mercury electrode.

Pulse Time (ms)	U. Peak (mV)	I. Peak (µA)	P. Peak (nW)	U. Width (mV)
0.2	-363	0.63	39.0	43
0.25	-340	1.51	153.2	61
0.3	-330	1.82	195.2	59
0.35	-333	1.97	212.4	62
0.4	-329	2.37	254.3	65
0.45	-330	2.52	270.3	58
0.5	-329	2.72	295.6	62
0.55	-333	2.76	299.6	64
0.6	-329	2.88	310.9	62
0.65	-332	2.90	302.8	59
0.7	-328	3.07	324.3	62
0.75	-329	3.26	339.1	53
0.8	-332	3.28	349.4	56
0.85	-330	3.45	375.5	54
0.9	-331	3.59	393.5	59
0.95	-330	3.76	401.3	59
1	-330	3.93	420.2	64

Appendix 2.4

Effect of the Modulation Frequency on the square-wave voltammetric determination of reduced glutathione (50×10^{-6} mol l^{-1}) at a dropping mercury electrode.

Mod. Frequency (Hz)	U. Peak (mV)	I. Peak (µA)	P. Peak (nW)	U. Width (mV)
50	-311	0.58	51.6	45
60	-315	0.85	81.8	46
70	-321	1.17	113.9	55
80	-320	1.48	150.5	59
90	-322	1.76	184	61
100	-323	2.05	213.7	57
120	-327	2.28	255.1	60
130	-328	2.3	249.3	58
135	-328	2.31	253.1	58
140	-327	2.41	261.8	60
145	-329	2.36	262.5	66
150	-328	2.37	262.3	64
155	-329	2.38	264.3	63
160	-329	2.39	271.6	65
180	-330	2.35	270.6	63
200	-333	2.2	256.6	68
220	-335	2.11	251.1	68
250	-335	1.88	226	72

Appendix 2.5

Effect of the Scan Rate (ms) on the square-wave voltammetric determination of reduced glutathione (50×10^{-6} mol l^{-1}) at dropping mercury electrode.

T. Measur.(ms)	U. Peak (mV)	I. Peak (µA)	P. Peak (nW)	U. Width (mV)
0.1	-320	1.63	165.5	51
0.2	-321	1.66	170.4	58
0.3	-322	1.69	169.7	52
0.4	-319	1.74	169.1	49
0.6	-320	1.78	171.6	55
0.8	-322	1.84	179.1	49
1.0	-324	1.88	191.7	59
1.2	-323	1.94	196.0	51
1.4	-322	2.02	201.4	53
1.6	-324	2.07	215.4	62
1.8	-324	2.14	230.7	46
2.0	-324	2.21	234.7	59
2.4	-323	2.30	250.5	56
2.6	-325	2.43	267.7	51
3.0	-325	2.47	273.5	73

Appendix 2.6

Calibration of a reduced glutathione ($x10^{-6}$ mol l^{-1}) in 2 x 10^{-2} mol l^{-1} citrate buffer pH 4.0 by square wave voltammetry at a dropping mercury electrode.

Concentration (μM)	U. Peak (mV)	I. Peak (μA)	P. Peak (nW)	U. Width (mV)
100	-340	3	325.2	62
	-344	2.99	327.1	63
	-340	2.98	326.0	64
	-340	3.00	328.0	62
50	-344	2.20	243.8	59
	-331	2.16	238.1	65
	-331	2.22	241.7	64
	-327	2.18	240.9	59
25	-317	1.37	168.1	57
	-316	1.35	166.9	65
	-319	1.41	170.1	57
	-316	1.39	170.4	59
20	-311	1.19	143.6	62
	-311	1.19	143.1	64
	-310	1.18	142.4	73
	-310	1.18	141.8	61
10	-326	0.81	119.7	79
	-297	0.80	96.1	54
	-299	0.76	96.7	31
	-305	0.74	96.2	72
5	-326	0.52	79.4	105
	-304	0.53	78.3	70
	-291	0.50	79.9	58
	-302	0.52	76.7	55
1	-324	0.33	57.2	40
	-329	0.35	59.3	127
	-295	0.61	54.2	30
	-313	0.34	58.2	34
0.1	-309	0.32	56.5	71
	-312	0.33	56.9	42
	-304	0.32	55.7	39
	-292	0.28	48.3	32

Appendix 2.7

Cyclic square-wave voltammetric calibration of reduced glutathione from 0.05 to 5×10^{-3} mol l^{-1},in 10^{-2} mol^{-1} citrate buffer pH 4.0 at hanging mercury electrode.

Concentration in GSH (mM)	Oxidising Peak (µA)
5	63.64
4	55.24
3	50.81
1	36.67
0.5	33.13
0.1	27.82
0.05	26.05
0	26.05

Appendix 2.8

Ultaviolet-Visible analysis @ 330 nm, of the glutathione/CDNB conjugate compound after elution through the immobilised GsT column.

CDNB Concentration (mol/l)	Absorbance @ 330 nm
8×10^{-5}	0.375
2×10^{-4}	0.685
3×10^{-4}	0.902
4×10^{-4}	1.182

Appendix 2.9

Peak Areas obtained for a range of 1-chloro-2,4-dinitrobenzene (0 to 0.8 x 10^{-3} mol.l^{-1}) in constant concentration of reduced glutathione (1 x 10^{-3} mol.l^{-1}) after elution through glutathione s-transferase reactor, analysed by high performance liquid chromatography, UV/Vis determination at 254 nm.

Mobile Phase : 49.5:49.5:1 % MeOH:H_2O:AcCOOH.

CDNB (mM)	Peak 1	SD	Peak 2	SD	Peak 3	SD
0	75244	435.5	149247	1319	219026	7390
0	74429		151885		233024	
0	75103		150587		221917	
0.1	34314	788.7	116676	5265.4	351846	25621
0.1	34420		124000		382970	
0.1	33004		126891		365059	
0.2	73534	2326.4	175410	5741.3	675703	33862
0.2	72499		172502		624463	
0.2	69088		164336		611732	
0.4	68560	1717	217324	13611	1308370	105680
0.4	71993		237328		1475745	
0.4	70156		243317		1503834	
0.6	77682	5603	326618	9445	1913469	78059
0.6	71215		327659		1996979	
0.6	66522		310804		1840990	
0.8	69263	425	495406	13453	1601223	47806
0.8	70113		506448		1507040	
0.8	69699		522176		1568393	
1	0	0	82004	6111.5	2432515	91727
1	0		81690		2447545	
1	0		92429		2598372	

Peak 1 : GSH

Peak 2 : CDNB

Peak 3 : CDNB/conjugate

CDNB (mM)	Peak 2	Peak 3	SD Peak 1	SD Peak 2
0	150573	224656	7390	1319
0.1	122522	366625	25621	5265.4
0.2	170749	637299	33862	5741.3
0.4	232656	1429316	105680	13611
0.6	321694	1917146	78059	9445
0.8	508010	2219914	47806	13453
1	85374	2492810	91727	6111.5

Appendix 2.10

Amperometric oxidation of a 10^{-3} mol.l^{-1} 1-chloro-2,4-dinitrobenzene and 1.5 x 10^{-3} mol.l^{-1} reduced glutathione in 10^{-2} mol.l^{-1} phosphate buffer pH 7.0, at + 0.9 V vs. Ag-AgCl, before elution through the aminopropyl porous glass beads glutathione s-transferase reactor respectively

a1) glassy-carbon electrode, a2) enzyme reactor / glassy-carbon,
b1) gold electrode, b2) enzyme reactor / gold,
c1) silver electrode, c2) enzyme reactor / silver electrode.

	Glassy-Carbon	Gold Electrode	Silver Electrode
Blank	465915	457033	494308
Blank	457243	448193	483898
Blank	458381	450908	468286
Average	**460513**	**452046**	**482164**
SD	**4713**	**4528**	**13097**
RSD (%)	**1.02**	**1.00**	**2.72**
Enzyme Reactor	305173	466856	358861
Enzyme Reactor	304856	420694	297525
Enzyme Reactor	303004	384334	313346
Average	**304344**	**423961**	**323244**
SD	**1172**	**41358**	**31843**
RSD (%)	**0.38**	**9.76**	**9.85**

Before - After (%)	**33.91**	**6.21**	**32.96**

Appendix 2.11

Determination of reduced glutathione (0.05 to 2 x 10^{-3} mol l^{-1}) in 10^{-2} mol l^{-1} phosphate buffer pH 7.0, at + 0.9 V vs. Ag-AgCl at glassy carbon electrode.

GSH concentration (x 10-3 mol l-1)	Peak Area (μV)
2	2122028
1	1108739
0.5	581346
0.2	218950
0.1	103643
0.05	26918

Appendix 2.12

Amperometric calibration of solutions of 1-chloro-2,4-dinitrobenzene from 0.1 to 0.8 x 10^{-3} mol l^{-1} in 10^{-3} mol l^{-1} reduced glutathione in 10^{-3} mol l^{-1} Tris.HCl, after elution through the glutathione s-transferase reactor. Determined at a glassy carbon electrode, at + 0.9 V vs. Ag-AgCl.

CDNB (mM)	Peak Area
0.1	48455200
	48495604
	49455044
0.2	45549252
	45068728
	45105040
0.3	44033232
	44952048
	45652800
0.4	39952184
	40010533
	39012456
0.5	28463852
	29383648
	29605132
0.6	22765264
	24193420
	24452636
0.7	20934150
	21819220
	21313446
0.8	11497264
	11558892
	11490702

CDNB (mM)	Av. Peak Area	SD	RSD (%)
0.1	48801949	565957	1.16
0.2	45241007	267565	0.59
0.3	44879360	812227	1.81
0.4	39658391	560156	1.41
0.5	29150877	605200	2.08
0.6	23803773	908666	3.82
0.7	21355605	444039	2.08
0.8	11515619	37619	0.33

Average RSD (%)	1.66